APPENDIX A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Soe *et al.*
Serial No. 10/676,006
Filed : October 2, 2003
Examiner: Wong, Leslie A
Group Art Unit: 1761
For: *A Method of Improving the Properties of a Flour Dough, a Flour Dough Improving Composition and Improved Food Products*

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF JØRN BORCH SØE UNDER 37 C.F.R. §1.132

I, **Jørn Borch Søe,** declare:

1. I am the co-inventor of the subject matter described and claimed in the above-captioned patent application.

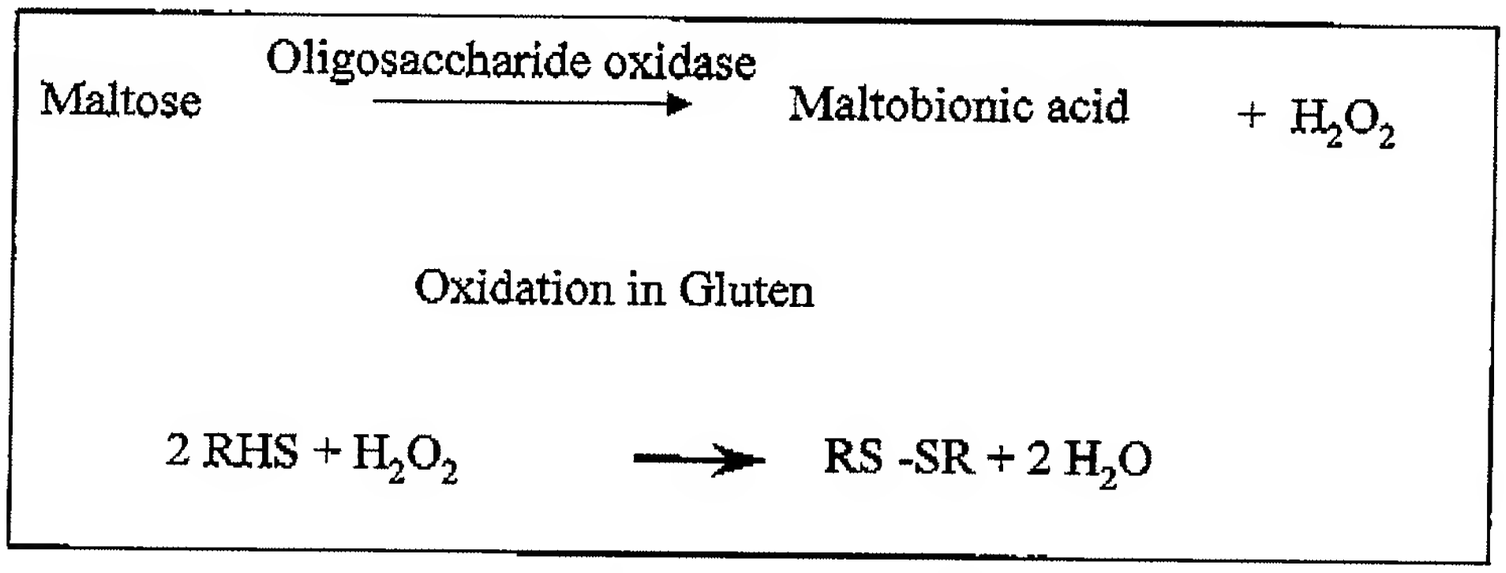
2. The following experimental data is presented on doughs produced using glucooligosaccharide oxidase ("GO"), an oxidoreductase that is known in the art and known to be capable of oxidizing maltose as of the priority date of the current application. See Lin *et al.*, "Purification and characterization of a novel glucooligosaccharide oxidase from *Acremonium strictum* T1," *Biochemica et Biophysica Acta*, Vol. 1118, p. 41-47 (1991) ("Lin *et al.*"), attached at Attachment A.

3. GO is an enzyme described before the priority date of the present application. GO is able to oxidize the mono-, di-and oligosaccharide with reducing end glucosyl linked by alpha- or beta-1,4 bonds. Lin *et al.* obtained the enzyme from a strain of *Acremonium strictum*, purified and characterized the enzyme for substrate specificity on different substrates. The enzyme was found to have highest relative activity on maltose but also showed high activity on oligosaccharides up to maltoheptose.

4. In a dough system, GO catalyzes the oxidation of glucose and glucooligosaccharides with formation of hydrogen peroxide, which is able to oxidize free thiol groups on gluten molecules resulting in formation of disulphide bridges in the gluten network and thereby create a stronger dough with improved rheological properties. Fig. 1 shows a GO reaction in a wheat flour dough. The formation of hydrogen peroxide

also contributes to improved dough handling properties and improved quality of the baked goods.

Fig. 1



5. In the following experiments, the effects of GO obtained by fermentation of a strain of *Acremonium strictum* on the oxidation of free thiol in wheat flour was tested. The microorganism used in this study was an *Acremonium strictum* from Centraalbureau voor Schimmelcultures, The Netherlands. The strain number was CBS 654,96. The organism was grown in a 3-L flask containing 100 g of wheat bran and 100 ml water, at 28°C. The flask was inoculated with approximately 1 ml of spore suspension containing 10^7 spores. After 8 days of growth on the wheat bran, 1000 ml 50 mM Tris-HCl buffer pH 7.8 was added to the flask, and soaked for 30 minutes at room temperature, and then squeezed through a cloth. The aqueous extract was centrifuged to remove particles. The supernatant was filter sterilized through a 0.2 μm filter (VacuCap 90 Filter Unit with 0.2 μm Supor Membrane) from Gelman Laboratory. After filtration, the filtrate (hereafter, "ferment") was frozen at –80°C and stored until analysis. The ferment containing GO activity was concentrated approximately 10 fold by ultrafiltration (10 kDa cutoff membrane). The concentrated ferment was loaded on a PD-10 column (Amersham Biosciences) equilibrated with a buffer containing 50 mM Tris-HCl pH 7.8. The same buffer was used for elution of the enzyme from the column. The PD-10 column was operated according to the manufacturer's instructions. This preparation is hereafter named "concentrated ferment." The GO activity was assayed essentially as described by

Lin *et al.* except that 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) was used instead of 4-aminoantipyrine and phenol as color reagent. The GO activity was measured by adding 10 µl of enzyme solution and 290 µl of substrate containing 0.20 g/L 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid), 10 mM maltose (or glucose or maltotriose), and 4 U/ml horse radish peroxidase (Sigma-Aldrich, St. Louis, USA, P-6782) to a microtiter well. The change in absorbency was measured at 405 nm at 30°C in a microtiter reader. The measured activity in mOD per minute was divided by 1680 (i.e. the slope of a standard curve generated by using H_2O_2 in the assay) to convert the activity to U per ml. One U produces 1 µmole of H_2O_2 per minute at the above described assay conditions. To measure the thiol groups in the dough, 6 g wheat flour, 120 mg sodium chloride and 15 ml demineralised water was agitated until a dough slurry was obtained. 600 µl dough slurry was transferred to a 3 ml centrifuge tube. 200 µl enzyme solution was added. The dough slurry was incubated at 30 °C on a vibration plate at 480 rpm for predefined time. 700 µl Ellman reagent was added (160 mg DTNB in 20 ml 3mM EDTA + 0.2 M Tris, pH 8.0 with HCl) and the tube was placed on a vibration plate at 30 °C for 10 minutes in the dark. The tube was centrifuged at 10000 g. for 10 minutes. 150 µl of the supernatant was transferred to a microtiter well. OD of the supernatant was measured at 412 nm against a blank. Free thiol in the flour was calculated based on a molar extinction coefficient of 13600 for NTB^{2-} (see Ellman, "A Colorimetric Method for Determining Low Concentration of Mercaptans," *Archives of Biochemistry and Biophysics*, 74, 443-450 (1958) and Chan *et al.*, "Direct Colorimetric Assay of Free Thiol Groups and Disulfide Bonds in Suspension of Solubilized and Particulate Cereal Proteins," *Cereal Chemistry*, 70 (1), 22-26 (1993), attached at Attachment B and C respectively).

6. The concentrated ferment was tested for activity with respectively glucose, maltose or maltotriose as substrate. The results are shown in Table 1. It is seen that the enzyme has significantly higher activity on maltose and maltotriose as substrate than on glucose. A sample of the concentrated ferment was heated at 95°C for 10 minutes in order to heat inactivate the enzyme. It is seen that the heat inactivated concentrated ferment does not show any activity in the assays. Also water was used as a blind sample

without showing any activity. These results confirm that the activities measured in the concentrated ferment are caused by enzyme activity.

Table 1

	Glucooligosaccharide oxidase activity [U/ml]		
Substrate	Concentrated ferment	Heat inactivated concentrated ferment	Water
Maltotriose	$8.3 \cdot 10^{-4}$	$-6 \cdot 10^{-5}$	$-5 \cdot 10^{-6}$
Maltose	$5.8 \cdot 10^{-4}$	$-4 \cdot 10^{-5}$	0
Glucose	$1.6 \cdot 10^{-4}$	$-5 \cdot 10^{-5}$	$1 \cdot 10^{-5}$

The ferment from *Acremonium strictum* CBS 654,96 was tested in a wheat flour slurry assay, and as a control the same ferment inactivated at 95°C for 10 minutes was tested. The effect of the enzyme on the amount of free reactive thiol groups was followed at different time intervals as shown in Table 2.

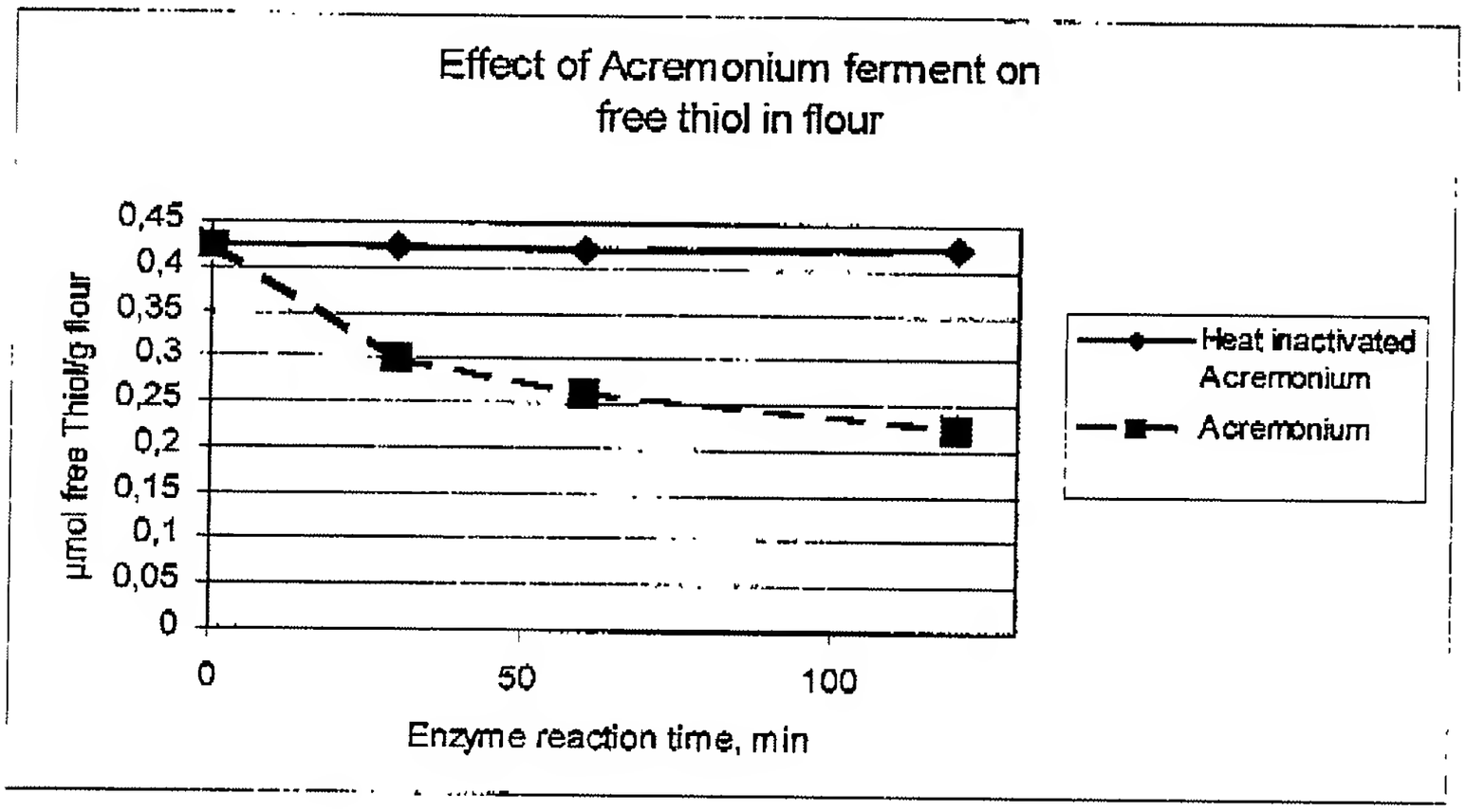
Table 2

	1	2	3	4	5	6
Wheat flour substrate, µl	600	600	600	600	600	600
CBS 654,96 ferment , µl	0	200	0	200	0	200
Heat inactivated ferment, µl	200	0	200	0	200	0
Water, µl	0	0	0	0	0	0
Reaction time, minutes	30	30	60	60	120	120

7. The amount of free reactive thiol in flour was analyzed by using Ellman reagent as mentioned above. The level of free thiol as a function of reaction time is illustrated in Fig. 2 which shows the effect of *Acremonium strictum* ferment of the amount of free thiol in flour gluten protein. The results clearly show that the GO from *Acremonium strictum* has an impact on the free thiol in the gluten protein. The same effect on thiol has also been observed when hexose oxidase is added to a dough slurry. It is also observed that the ferment inactivated at 95°C for 10 minutes does not have any effect on the level of free thiol. This confirms that the enzyme is inactivated and that the thiol oxidation is caused by an enzymatic reaction, which can be deactivated by heating.

In a parallel experiment (results not shown), water was added instead of *Acremonium* ferment. This experiment confirms that the level of reactive thiol does not change as a function of time, indicating the reduction in the amount of free thiol when *Acremonium* ferment is added to a flour slurry can only be explained by the enzymatic reaction.

Fig. 2



8. In another experiment the ferment of *Acremonium strictum* CBS 654,96 was concentrated by ultra filtration. The concentration factor was approximately factor 10. The effect on thiol in gluten of the concentrated ferment was tested in a flour slurry assay. The concentrated ferment was tested in different dilution concentrations and the reaction time was 30 minutes in this experiment (Table 3). As a control, the same concentration of heat inactivated concentrated ferment was also tested.

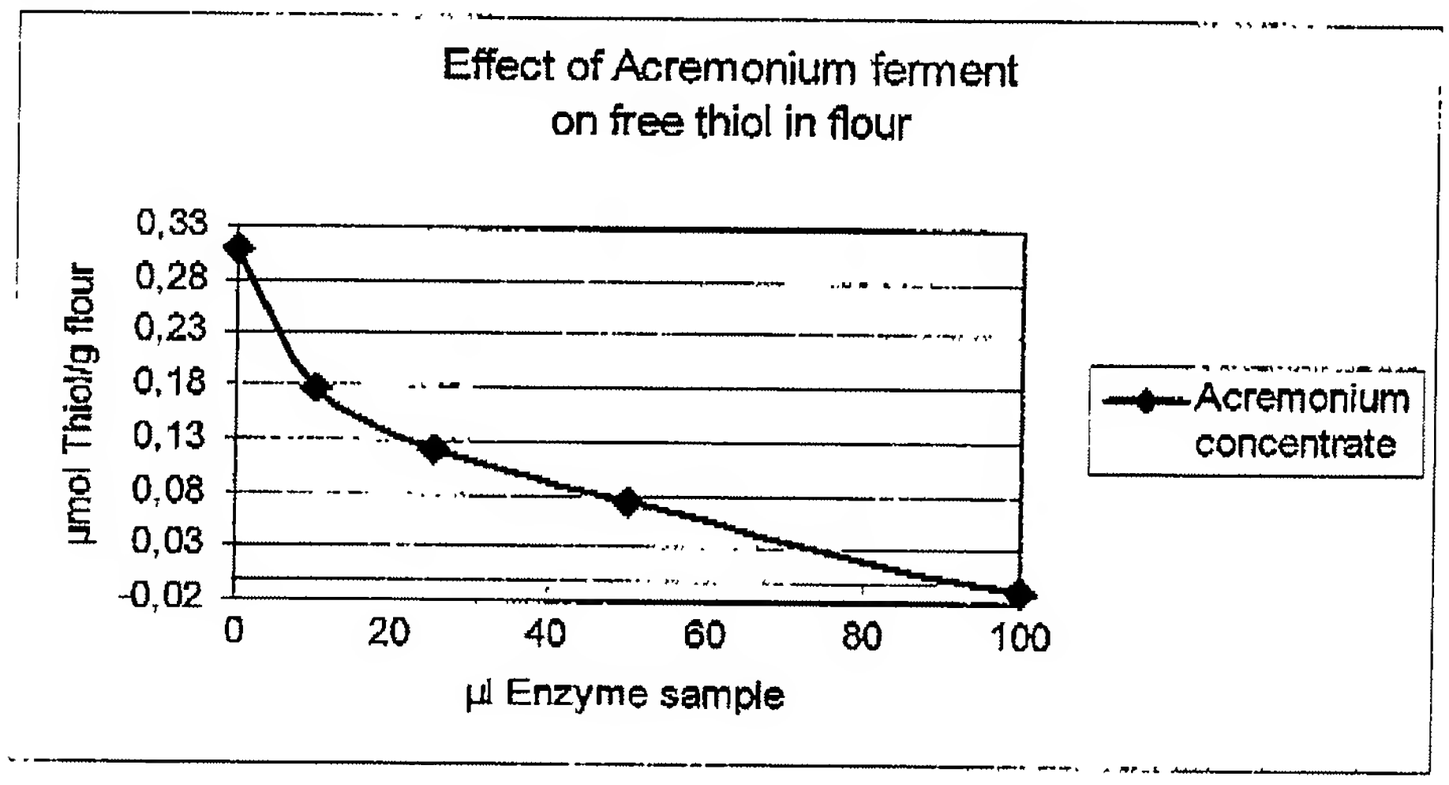
Table 3

	1	2	3	4	5	6	7	8
Wheat flour substrate, µl	600	600	600	600	600	600	600	600
Concentrated ferment, µl	10	25	50	100				
Heat inactivated concentrated ferment, µl					10	25	50	100
Water, µl	190	175	150	100	190	175	150	100
Enzyme reaction time, minutes	30	30	30	30	30	30	30	30

The amount of free reactive thiol was measured according to the procedure mentioned above, and the decrease in the amount of free thiol in the dough slurry relative to the

control sample containing the heat-inactivated sample was calculated (Fig. 3). Fig. 3 shows the effect of concentrated *Acremonium strictum* ferment on free reactive thiol in flour. The results clearly illustrate that the concentrated ferment from *Acremonium strictum* results in oxidisation of free thiol in a dough slurry. Fermentation of *Acremonium strictum* CBS 654,96 produced a ferment containing an oxidoreductase. Analysis on different substrates showed that this ferment was more active on maltose and maltotriose than on glucose. It was therefore concluded that the ferment contains a GO. The ferment was tested in wheat flour dough slurry, and it was confirmed that the ferment contains an enzyme, which action results in oxidation of free thiol groups in wheat gluten. It is therefore concluded that the GO from *Acremonium strictum* is responsible for oxidization of free thiol groups in wheat dough. The oxidation of free thiol groups of gluten protein molecules results in the formation of disulphide bridges in the gluten network and thereby creates a stronger dough with improved rheological properties. GO from *Acremonium strictum* is thus another example of an oxidoreductase which is able to oxidize maltose that has beneficial effects on rheological properties of a dough.

Fig. 3



9. All statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: May 23, 2008

Jørn Borch Søe

Jørn Borch Søe

ATTACHMENT A

Biochimica et Biophysica Acta, 1118 (1991) 41–47

BBAPRO 34049

Purification and characterization of a novel glucooligosaccharide oxidase from *Acremonium strictum* T1

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Key words: Glucooligosaccharide oxidase; Enzyme purification; Enzyme characterization; Fungus; (*Acremonium*)

A novel glucooligosaccharide oxidase was purified 495-fold from wheat bran culture of a soil-isolated *Acremonium strictum* strain T1 with an overall yield of 21%. This enzyme was composed of a single polypeptide chain with a molecular mass of 61 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and size-exclusion high-performance liquid chromatography. Its isoelectric point was pH 4.3–4.5. This enzyme contained 1 mol of FAD per mol of enzyme and showed absorption maxima at 274, 379 and 444 nm. This enzyme was stable in the pH range of 5.0 to 11.0 with an optimal reaction pH of 10.0. The optimal reaction temperature was 50°C. It was stable up to 50°C for 1 h at pH 7.8. This enzyme oxidized those oligosaccharides with glucose residue on the reducing end and each sugar residue jointed by α or β-1,4 glucosidic bond. The relative activity of this enzyme toward maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, lactose, cellobiose and glucose was 100:94:74:46:66:56:64:47:59. To our knowledge, this is the first report on the discovery of an glucooligosacharide oxidase as judged from enzyme substrate specificity.

Introduction

Enzymes that catalyze the oxidation of glucose and other sugars directly to the corresponding aldonic acids appear to be widely distributed in nature, since they have been found in bacteria, fungi, algae and animal tissues. Sugar dehydrogenase systems of genus *Pseudomonas* [1], *Gluconobacter* [2] and *Bacillus* [3,4] were examined in detail. Although most of the sugar dehydrogenases were specific to monosaccharides, there were a few disaccharide dehydrogenases, such as the lactose dehydrogenase of *Pseudomonas* [5] and the maltose dehydrogenase of alkalophilic *Corynebacterium* [6], which were specific for disaccharides. The galactose dehydrogenase purified from rat liver [7] also reacted with maltose and cellobiose. Similarly, most of the known sugar oxidases were specific to monosaccharide except the galactose oxidase of *Polyporus circinatur* [8] which exhibited a much higher affinity for galactosides and polysaccharides containing galactose end groups. None of the sugar oxidases hitherto reported showed high reactivity toward maltooligosaccharides, which are composed of α-D-glucopyranosyl units jointed by 1 → 4 bond. The relative activities of hexose oxidase of red alga *Chondrus crispus* [9] and glucose oxidase of *Iridophycus flaccidum* [10] toward glucose and maltose were 100:40 and 100:47, respectively. Recently, we conducted a screening program for sugar oxidase-producing microorganisms from soil. Strain T1, identified as *Acremonium strictum*, was isolated. The oxidase purified from the aqueous extract of a wheat bran culture of this strain showed high reactivities toward maltose, lactose, cellobiose and maltooligosaccharides composed of up to seven glucose units. This new oxidase was named glucooligosaccharide oxidase based on its substrate specificity. We here report its purification and some of its properties.

Materials and Methods

Materials

Fractogel DEAE-650 was purchased from Merck Darmstadt (Germany). Toyopearl Phenyl-650M and

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TSK G3000SW column were from Toyo Soda Manufacturing (Japan). Biogel P-100 and SDS-PAGE molecular weight standards were from Bio-Rad Laboratories (U.S.A.). Ultrogel-HA was from IBF biotechnics (France). Isoelectric focusing calibration kit, ampholine pagplate for isoelectric focusing and concanavalin A Sepharose 4B were from Pharmacia LKB biotechnology (Sweden). Endoglycosidase F was from Du Pont NEN (U.S.A.). 4-Aminoantipyrine was from Wako Pure Chemical Industries (Japan). Phosphodiesterase was from Amano Phamaceutical (Japan). Molecular weight markers for gel-filtration chromatography, peroxidase, pronase, and all the sugars were from Sigma (U.S.A.). All other chemicals were of analytic reagent grade.

Microorganism and culture conditions

A fungal strain, T1, was isolated from soil and was identified as *Acremonium strictum*. For enzyme production, the organism was grown in 3-l flasks, each of which contained 100 g of wheat bran supplemented with 100 ml of water, at 28 °C. After 7–10 days of cultivation, the entire culture was used for enzyme preparation.

Enzyme assay

The glucooligosaccharide oxidase activity was estimated by peroxidase chromogen method. An aliquot of enzyme was incubated at 30 °C in 1 ml of 50 mM Tris-HCl buffer (pH 7.8, buffer A) containing 10 mM maltose, 2 units peroxidase, 0.1 mM 4-aminoantipyrine and 1 mM phenol. The increase of optical density was followed at 500 nm for 3 min. Otherwise, the glucooligosaccharide oxidase activity was estimated by monitoring the consumption of oxygen with a oxygraph (Oxy-5, Gilson Medical Electronics). The reaction was initiated by the addition of appropriate amount of enzyme to the reaction mixture containing 10 mM maltose in buffer A, and the initial velocity of oxygen consumption was measured.

One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of H_2O_2 or consumed 1 μmol of O_2 per min at 30 °C.

Protein determination

Protein was determined by the method of Lowry et al. [11] with bovine serum albumin as a standard.

Enzyme purification

A typical purification scheme of the glucooligosaccharide oxidase from the wheat bran culture extract is described below. All the operations were carried out at 4 °C.

Preparation of crude extract. 700 g of wheat bran culture of strain T1 was soaked in 2.5 l of buffer A for 30 min at room temperature and squeezed through a cloth. The aqueous extract was centrifuged to remove particles.

Ammonium sulfate fractionation. The clarified aqueous extract (2.3 l) was brought to 45% saturation ammonium sulfate and the precipitate was removed centrifugation. Ammonium sulfate was added to supernatant liquid to 80% saturation. The resu precipitate was collected by centrifugation and dissolved in a minimal volume of buffer A. The enz solution was dialyzed overnight against the same bu

Fractogel DEAE-650M column chromatography. dialyzed 45–80% ammonium sulfate fraction (290 was applied to a Fractogel DEAE-650M column (6 cm). After the column was washed with 1.2 l buffe containing 80 mM NaCl, the enzyme was then el with buffer A containing 170 mM NaCl. The flow was 300 ml/h.

Toyopearl Phenyl-650M column chromatogra Ammonium sulfate was added to the pooled ac fractions to a final concentration of 2 M. The enz solution was then applied to a Toyopearl Phenyl-65 column (3.5 × 7 cm) which was preequilibrated v buffer A containing 2 M ammonium sulfate. After column was washed with 200 ml of buffer A contair 2 M ammonium sulfate, it was eluted with a 1 l lin gradient of 2 to 0 M ammonium sulfate in buffer The flow rate was 140 ml/h. The time period for gradient elution was 7.1 h. In order to concentrate pooled active fractions (470 ml), ammonium sulf was added to the enzyme solution to a final concent tion of 2 M and then recharged to a small Toyope Phenyl 650M column (1 × 6 cm) which was preeq librated with buffer A containing 2 M ammoniu sulfate. At this time, the enzyme was eluted direc with buffer A. The volume of pooled active fractic was 15 ml.

Biogel P-100 column chromatography. The enzyr solution was applied to a Biogel P-100 column (2.5 115 cm). The column was eluted with buffer A at flow rate of 15 ml/h.

Ultrogel-HA hydroxyapatite column. The pooled a tive fractions was further purified by passing it throu a Ultrogel-HA column (2 × 25 cm), which was preequ librated with 10 mM phosphate buffer (pH 7.8). Aft washing with 10 mM phosphate buffer, the column w eluted with a 1 l linear gradient of 10 to 500 m phosphate buffer. The flow rate was 96 ml/h. The tim period for the gradient elution was 10.4 h. The activ fractions were pooled and stored at −20 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophore sis (SDS-PAGE)

The method was carried out on 10% acrylamid slabs using modified Laemmli buffer system [12] Coomassie brilliant blue R-250 was used for staining.

Polyacylamide gel isoelectric focusing

Isoelectric focusing was carried out with an LKE multiphor II electrophoresis unit at 4 °C for 2 h. A

ready made polyacrylamide gel (ampholine pagplate) with pH range of 4.0–6.5 was used. The anode and cathode solutions used were 0.1 M glutamic acid in 0.5 M H_3PO_4 and 0.1 M β-alanine, respectively. The pH gradient was determined by the migration of the *pI* markers and by a surface pH electrode.

Periodic acid-Schiff staining

Periodic acid-Schiff staining for glycoprotein was performed as described by Zacharius et al. [13].

Molecular weight determination by high-performance liquid chromatography

The apparent molecular weight of the enzyme was determined by high-performance liquid chromatography (Waters, U.S.A., composed of a pump model 510, a tunable absorbance detector model 486 and a U6K injector) on a molecular sieve column (TSK-gel G3000SW XL (7.5 × 600 mm), Toyo Soda, Japan). The detection was using UV absorption at 280 nm. The eluent used was buffer A containing 0.5 M NaCl. The flow rate and the column temperature were 0.5 ml per min and 25 °C, respectively.

Amino acid composition analysis

The enzyme was hydrolyzed with 6 M HCl in a sealed evacuated glass tube at 110 °C for 24, 48, 72 and 98 h. The hydrolysates were analyzed with an amino acid analyzer (Beckman 6300). The content of cysteine plus cystine was determined by the method of Moore [14]. The content of tryptophan was estimated by hydrolyzing the protein with 4 N methanesulfonic acid as described by Liu and Moore [15].

Results

Purification of glucooligosaccharide oxidase

Table I summarizes the results of the purification of glucooligosaccharide oxidase described under Materials and Methods. The enzyme was purified 495-fold, with a recovery of 21%, from the wheat bran culture extract. The specific activity of the purified enzyme was 2.2 units per mg of protein. The final enzyme preparation was judged to be homogeneous by SDS-PAGE

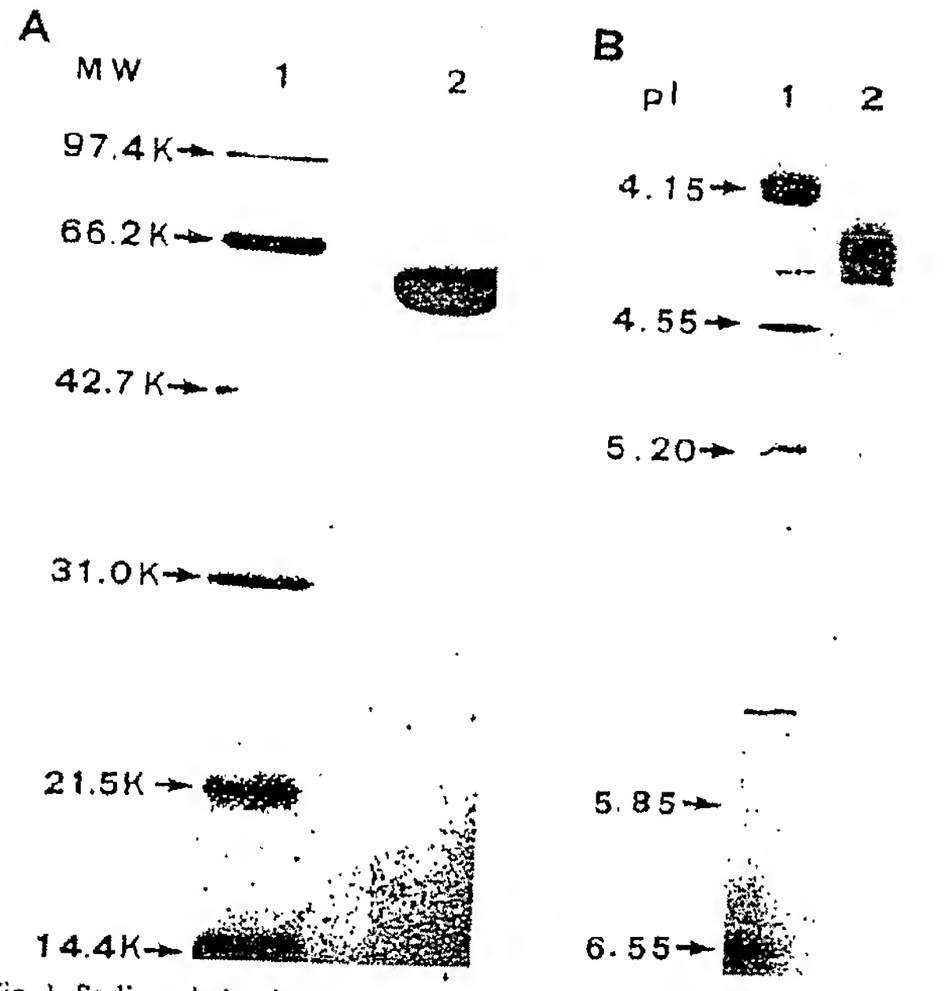

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) and isoelectric focusing (B) of the glucooligosaccharide oxidase. (A) The gel concentration was 12%. Lane 1, molecular mass markers: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Lane 2, 20 μg of purified enzyme. (B) Lane 1, *pI* markers: glucose oxidase (*pI* 4.15), soybean trypsin inhibitor (*pI* 4.55), β-lactoglobulin A (*pI* 5.20), bovine carbonic anhydrase B (*pI* 5.85) and human carbonic anhydrase B (*pI* 6.55). Lane 2, 5 μg of purified enzyme.

(Fig. 1A). The enzyme activity was increased after Toyopearl phenyl-650M chromatography. This might be due to the removal of some unknown inhibitory factors from the enzyme preparation in this step.

Molecular weight

The apparent molecular weight of the purified enzyme was 61 000 as determined by SDS-PAGE (Fig. 1A) and size-exclusion HPLC on a G3000SW column (Fig. 2). These results suggested that the enzyme was composed of a single polypeptide chain.

TABLE I

Purification of the glucooligosaccharide oxidase

Step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2300	12400	51	0.004	100	1
$(NH_4)_2SO_4$ precipitation	290	3150	38	0.012	75	3
Fractogel DEAE-650	1560	1160	27	0.023	53	6
Toyopearl phenyl-650	470	326	35	0.11	69	27
Biogel P-100	57	102	20	0.20	39	49
Ultragel-HA	107	5	11	2.20	21	495

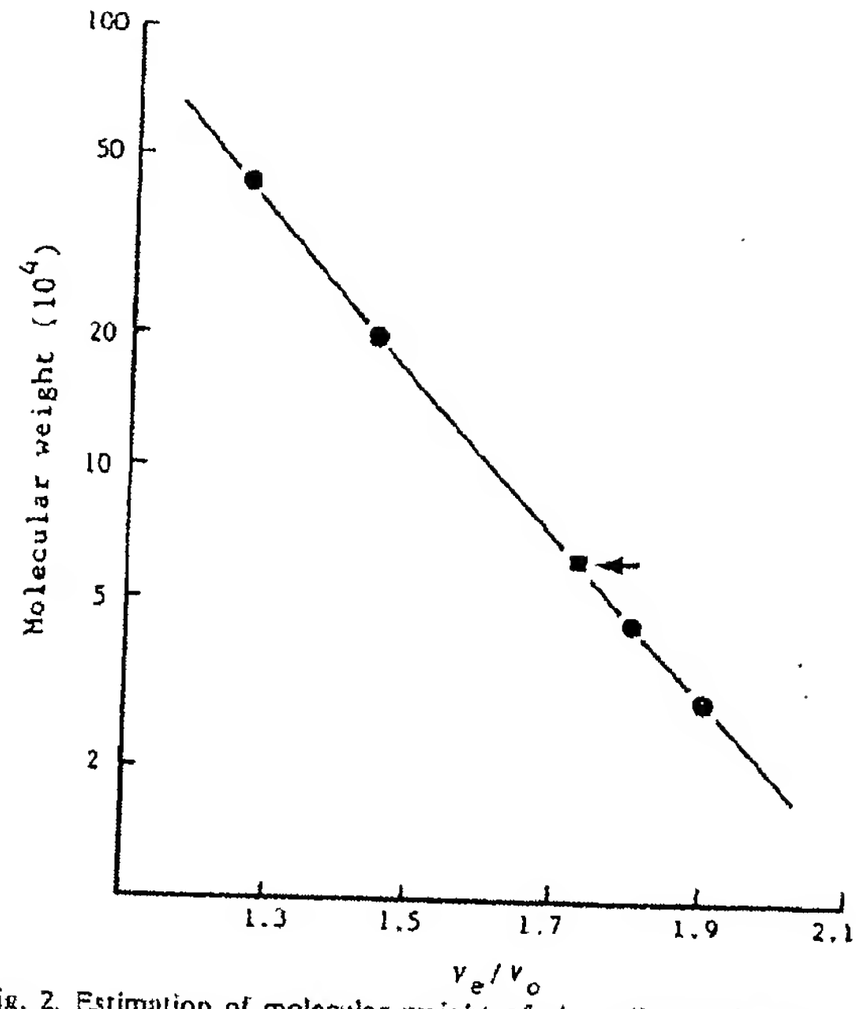

Fig. 2. Estimation of molecular weight of glucooligosaccharide oxidase by high-performance liquid chromatography on a molecular sieve column (7.5 by 600 mm, TSK G3000SW). The molecular weight markers used were apoferritin (443 kDa), β-amylase (200 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The position of the glucooligosaccharide oxidase is indicated by the arrow. For details, see Materials and Methods.

Isoelectric point

The glucooligosaccharide oxidase was separated into five bands on isoelectric focusing (Fig. 1B), and their isoelectric points were estimated to be between pH 4.3–4.5. Since the enzyme preparation was shown to be homogeneous by SDS-PAGE (Fig. 1A) and by N-terminal sequence analysis (Scheme 1), the five bands might represent isoforms of the enzyme with different degrees of glycosylation.

Amino acid composition

The amino acid composition of the enzyme is given in Table II.

Amino-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the first 30 residues of the glucooligosaccharide oxidase was deter-

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                5                  10
Asn-Ser-Ile-Asn-Ala-(X)-Leu-Ala-Ala-Ala-
               15                  20
Asp-Val-Glu-Phe-His-Glu-Glu-Asp-Ser-Glu-
               25                  30
Gly-Lys-Asp-Met-Asp-(X)-Thr-Ala-Phe-Asn-
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Scheme 1. Amino-terminal sequence of the glucooligosaccharide oxidase. (X), undefined residues.

TABLE II

Amino acid composition of the glucooligosaccharide oxidase

Amino acid residue	mol/100 mol
Asx	12.9
Thr	6.2
Ser	5.5
Glx	11.0
Pro	3.2
Ala	10.2
Gly	10.7
Val	6.0
Met	1.3
Ile	4.6
Leu	7.7
Tyr	4.6
Phe	4.1
His	2.2
Lys	3.8
Trp	3.1
Arg	2.3
Cys	0.8

mined by gas-phase sequencing (Scheme 1). Non the reported protein sequence was found to show sequence homology with the N-terminal sequence this enzyme after a computer-aided protein seque homology search was performed.

Absorption spectrum

The absorption spectra of the purified enzy showed maxima at 274, 379 and 444 nm with mc absorption coefficients of 117400, 12540 and 104 respectively. The addition of maltose or sodium hyd sulfite to the enzyme solution made the peak at 444 disappear (Fig. 3). These spectral characteristics su gested that the enzyme is a flavoprotein. The $a^{1\%}_{1\,cm,\,nm}$ value of this enzyme was calculated to be 19.2.

Prosthetic group

The presumed flavin group of this enzyme boun tightly with the apoenzyme and was not released fro enzyme after denaturing enzyme by boiling or treati with 25% trichloroacetic acid. The enzyme was d gested with pronase and then incubated with phosph diestearase. The released AMP was identified by thi layer chromatography on cellulose plate with butanol : acetic acid : H_2O = 2 : 1 : 2 (v/v) as solven This result suggested that the enzyme contained FAI but not FMN as the prosthetic group. From the ab sorbance of the pronase digested-enzyme solution a 450 nm, the amount of FAD was determined to be mol per mol of enzyme.

Enzyme as glycoprotein

Two pieces of evidence indicate that the glucooligosaccharide oxidase is a glycoprotein. First, the

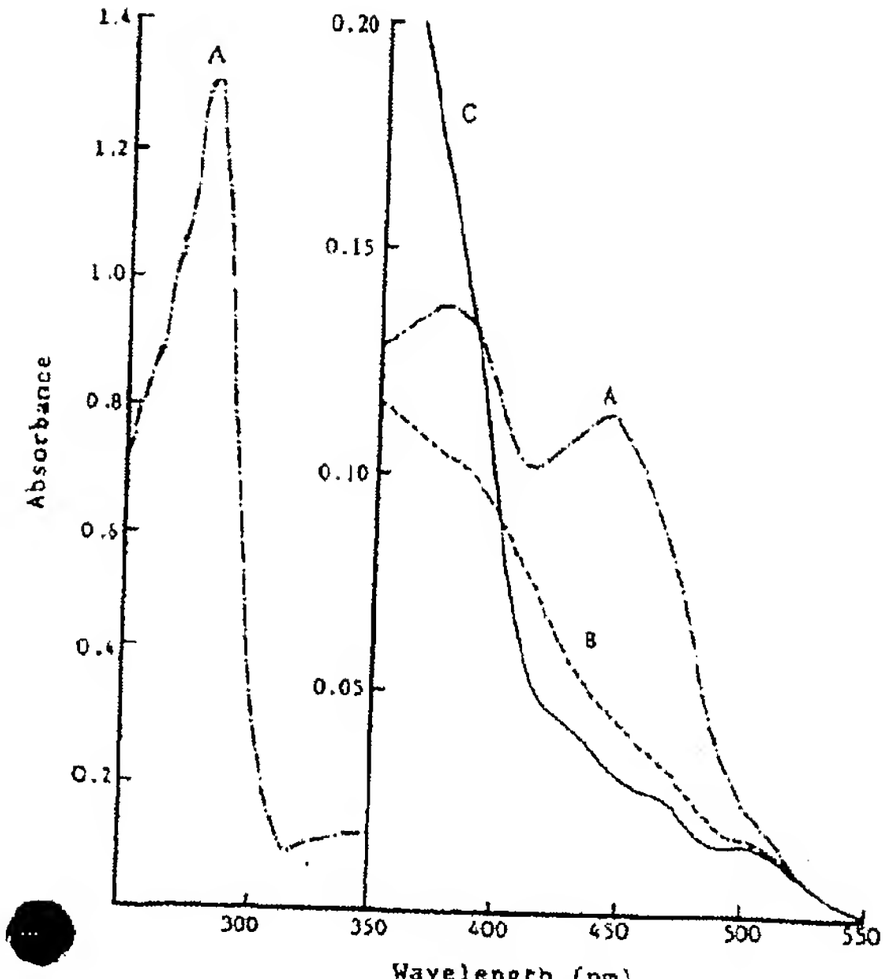

Fig. 3. Absorption spectrum of glucooligosaccharide oxidase (0.66 mg/ml) in buffer A. Absorption spectrum was taken with a Perkin-Elmer lambda 15 UV/VIS spectrophotometer, with a 1.0-cm light path. (A) native enzyme, (B) the prosthetic group of enzyme was reduced by adding maltose to a concentration of 400 mM, (C) the prosthetic group of enzyme was reduced by adding sodum hydrosulfite to a concentration of 50 mM.

enzyme was positively stained with periodate-Schiff's reagent on SDS-PAGE. Second, it bound to concanavalin A-Sepharose 4B and was eluted by buffer containing 0.4 M methyl-α-D-glucopyranoside.

Optimal reaction pH and pH stability

As judged from the bell-shaped pH versus activity profile shown in Fig. 4A, the optimal reaction pH of this enzyme was around 10. The enzyme was very ble in the pH range from 5.0 to 11.0 (Fig. 4B).

Optimal reaction temperature and thermal stability

As shown in Fig. 5A, the enzyme activity reached a maximum at about 50 °C under standard assay conditions at temperatures varying from 20 to 70 °C. Thermal stability was investigated by incubating the enzyme in buffer A at a designated temperature for 1 h. The enzyme was stable up to 50 °C (Fig. 5B).

Substrate specificity

The ability of the enzyme to catalyze oxidation of various sugars was investigated (Table III). Glucose was oxidized by this enzyme. All the other D-form monosaccharides tested, including lyxose, arabinose, ribose, xylose, fructose, allose, altrose, galactose, guose, idose, mannose, tagatose and sedoheptulose, were

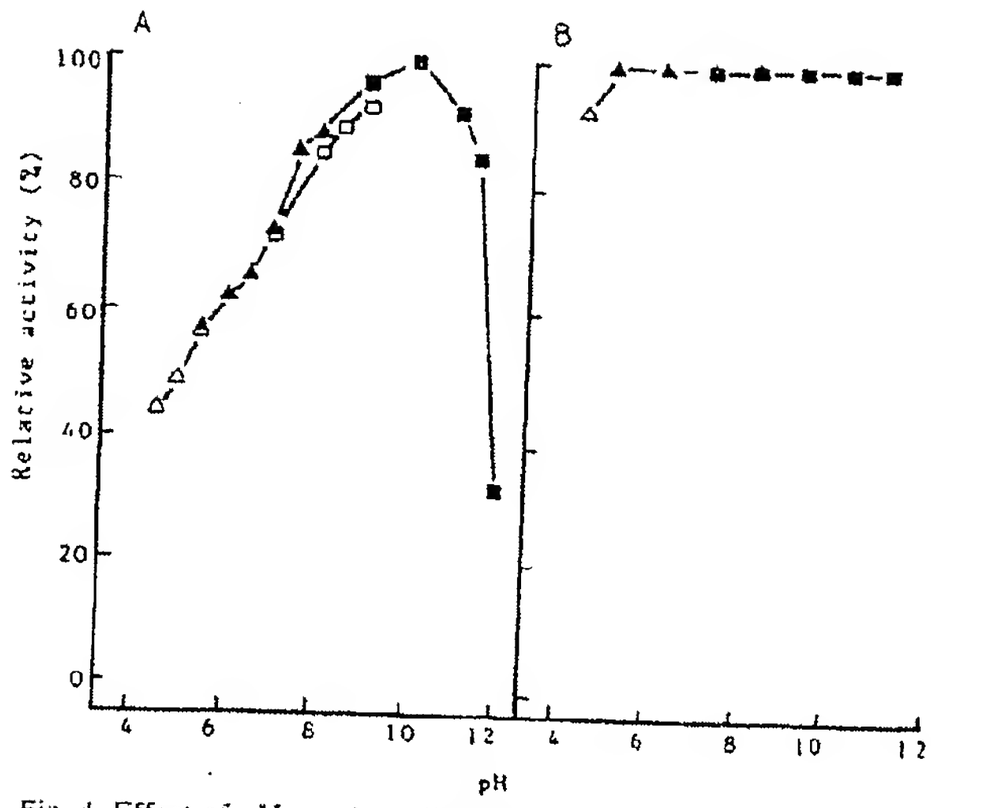

Fig. 4. Effect of pH on glucooligosaccharide oxidase activity (A) and stability (B). (A) Enzyme activity was assayed in various buffers at the pH values indicated by oxygraph method as described in the text. (B) Enzyme (15 μg/ml) was incubated in various buffers at the pH values indicated. After incubation at 30 °C for 1 h, the residual activity was estimated at pH 7.8 by peroxidase-chromogen method as described in the text. The buffer system (50 mM) used were CH_3COOH-CH_3COONa (pH 4.5–5.5) (△), KH_2PO_4-KOH (pH 8.0–9.0) (▲), Tricine-HCl (pH 7.0–9.0) (□) and Na_2CO_3-$NaHCO_3$ (pH 9.0–12.0) (■).

not oxidized. Derivatives of the D-form monosaccharides, such as methyl-glucopyranoside, 2-deoxy-glucose, fucose, rhamnose, glucose-1-phosphate, glucose-6-

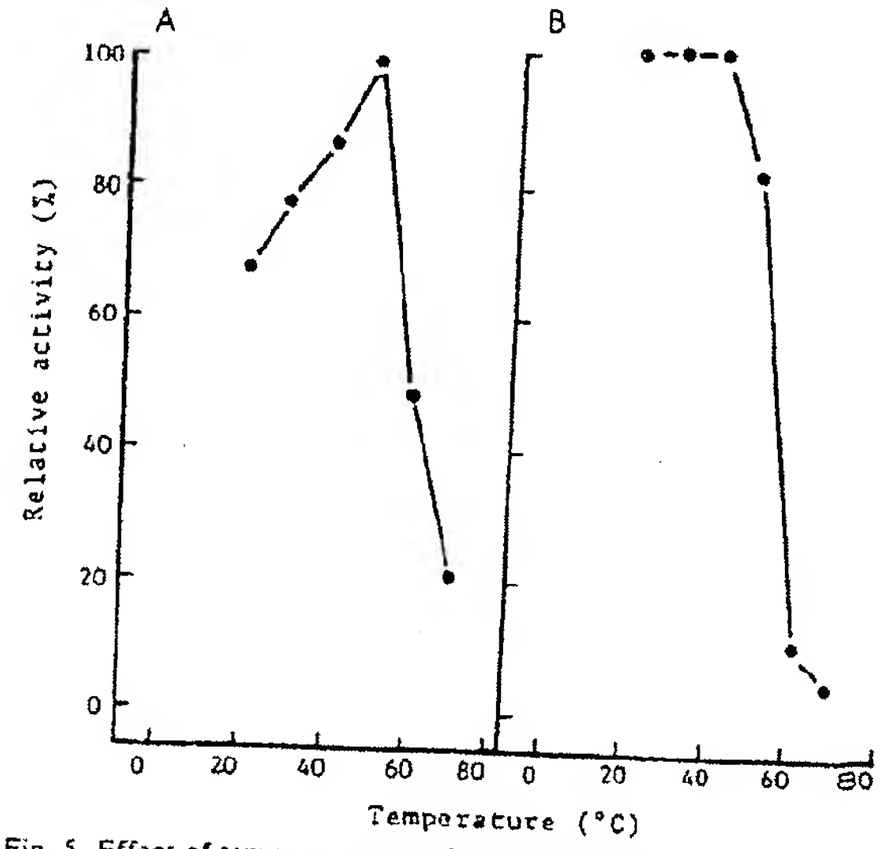

Fig. 5. Effect of temperature on glucooligosaccharide oxidase activity (A) and stability (B). (A) Enzyme activity was assayed by oxygraph method as described in the text at various temperature in buffer A. (B) Enzyme (15 μg/ml in buffer A) was incubated at the temperature indicated for 1 h, and the remaining activity was estimated at 30 °C by peroxidase-chromogen method as described in the text.

TABLE III

Substrate specificity of glucooligosaccharide oxidase

Enzyme activity was measured by peroxidase-chromogen method as described in the text. All values represent the percentage of activity as compared to the value obtained with maltose

Substrate	Relative activity (%)
Maltose	100
Maltotriose	94
Maltotetraose	74
Maltopentaose	46
Maltohexaose	66
Maltoheptaose	56
Glucose	59
Lactose	64
Cellobiose	47

phosphate, glucosamine, galactosamine, *N*-acetylglucosamine, gluconic acid and glucuronic acid, or sugar alcohols, such as myoinositol, xylitol, sorbitol, mannitol and arabitol, were not oxidized by this enyzme.

As for the disaccharides, maltose and cellobiose, in which two glucose units are jointed by an α or β-1,4-glycosidic linkage and lactose, which consists of galactose jointed to glucose by a β-1,4 linkage, were good substrates for the enzyme. Neverthless, disaccharides containing other types of linkage, such as nigerose (Glc-α[1 → 3]-Glc), laminaribose (Glc-β[1 → 3]-Glc), isomaltose (Glc-α[1 → 6]-Glc), gentibiose (Glc-β[1 → 6]-Glc), trehalose (Glc-α[1 → 1]-Glc), sucrose (Glc-[α1 → β2]-Fru), and melibiose (Gal-α[1 → 6]-Glc) were not oxidized by this enzyme at all.

Moreover, this oxidase reacted with maltooligosaccharides containing up to seven monosaccharide units. If the reactivity of this enyzme toward maltose was taken as 100%, the relative activity of this enzyme toward maltoheptaose could reach 56%.

In general, this novel glucooligosaccharide oxidase oxidized those oligosaccharides with glucose unit on the reducing end and each sugar unit were jointed by α or β-1,4 linkage.

TABLE IV

Effect of metal ions and chelating agents on glucooligos oxidase acticity

Enzyme (15 μg) was incubated in buffer A contalning vari ions or chelating agents for 15 min at 30 °C. The residual were assayed by oxygraph method as described in text

	Concentration (mM)	Relative activi (%)
Control	0	100
$HgCl_2$	1	6
$FeCl_2$	0.5	0
$FeCl_3$	0.5	80
$PbCl_2$	1	88
$CoCl_2$	1	93
$NiCl_2$	1	83
$CdCl_2$	1	93
$BaCl_2$	1	83
$SrCl_2$	1	79
$LiCl_2$	1	91
$CaCl_2$	1	92
$MgCl_2$	1	84
$CuCl_2$	1	103
$ZnCl_2$	1	106
EDTA	1	91
O-phenanthrolin	1	98

Effects of metals and metal chelating agents

The effects of various metal ions and inhibito the enzyme activity are shown in Table IV. Th zyme activity was potently inhibited by 1 mM Hg^{2+} and Fe^{2+}. Metal chelating agents, EDTA an phenanthroline, did not show a significant inhit effect to the enzyme activity.

Discussion

According to the substrate specificity shown in T III, the glucooligosaccharide oxidase purified wheat bran culture of *Acremonium strictum* T1 i deed a new type of oxidase. Maltose was the fav substrate, followed by maltotriose. As for maltoi taose, the largest substate available commercially,

TABLE V

Some properties of saccharide oxidases from different microorganisms

	Glucooligosaccharide oxidase	Glucose oxidase	Galactose oxidase	Pyranose oxidase
Source	*Acremonium strictum*	*Penicillium notatum*	*Polyporus circinatus*	*Basidiomycetous sp.*
Molecular weight	61000	150000	42000	69000
Isoelectric point	4.3	< 4.5	–	6.3
Optimal pH	10	5.6	7.0	5–8
Optimal temperature	50° C	40° C	30° C	60° C
Prosthetic group	FAD	FAD	Cu^{2+}	FAD
Substrate specificity	$(glucose)_n$, (n = 1–7) cellobiose, lactose	glucose, mannose, 6-methyl glucose	galactose, guaran. raffinose, stachyose	glucose, xylose, 6-deoxyglucose

enzyme still possessed about 56% reactivity as compared with that for maltose. None of the sugar oxidases hitherto reported showed reactivity toward maltooligosaccharides containing more than three glucose units. We thus believe that this is the first report on the discovery of a glucooligosaccharide oxidase.

Table V summarized the general properties of the glucooligosaccharide oxidase of *A. strictum* T1 together with those of glucose oxidase from *Penicillium notatum* [16], galactose oxidase from *Polyporus circinatus* [8] and pyranose oxidase from *Basidomycetous* [17]. The glucooligosaccharide oxidase of *A. strictum* T1 was composed of a single polypeptide chain with molecular weight of 61 000. This enzyme was found to be a glycoprotein similar to glucose oxidase and pyranose oxidase. A most surprising characteristic of this enzyme is its high optimal reaction pH (pH 10) which is very different from those of other fungi oxidases, including the three oxidases listed in Table V. These enzymes all have their optimal reaction pHs in weakly acidic range. The other significant difference between the current enzyme and other oxidases is their substrate specificity. Our enzyme had high activity toward maltose and maltotriose, whereas the other three oxidases exhibited low selectivity against disaccharides and oligosaccharides.

The glucooligosaccharide oxidase might be useful for the quantitative determination of maltose, lactose and cellobiose, since it showed high activity and specificity for these sugars. This enzyme might also be useful for the activity assays of amylase and cellulase, since the reaction product of the amylase and cellulase are maltose and cellulobiose which are the favorite substrates of this enzyme. We also expect that this enzyme could be used for the production of several oligosaccharic acids, such as maltobionic acid which is the reaction product derived from maltose.

Acknowledgements

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ATTACHMENT B

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A Colorimetric Method for Determining Low Concentrations of Mercaptans

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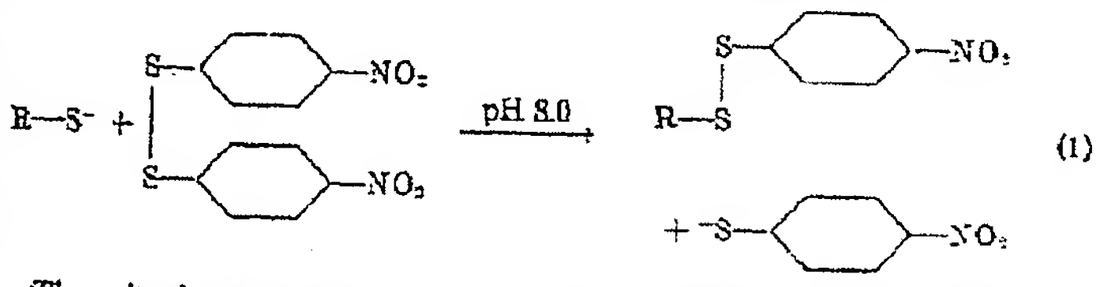
Received July 25, 1957

INTRODUCTION

Chinard and Hellerman (1) have written a comprehensive review on the determination of mercaptans in which they emphasize the difficulties in these measurements. Of the colorimetric methods, these authors state: "The precision . . . does not appear to be of a very high order"; also, "The nitroprusside test is quite unspecific for —SH groups."

The method for tissue-staining described by Seligman (2) involves the interchange of an aromatic disulfide with tissue mercaptans. If the residual thiophenol is colored, this reaction becomes the basis of a simple method for mercaptan determination. Bis(*p*-nitrophenyl)disulfide (PNPD) reacts quantitatively with many mercaptans to release 1 mole of *p*-nitrobenzenethiol per mole of mercaptan. The reaction is:



(1)

The nitrobenzenethiol anion is yellow, having a molar extinction coefficient $\left(\frac{1}{c}\log\frac{I}{I_0}\right)$ of 13,600/cm. at 412 mμ (see Fig. 1). *p*-Nitrobenzenethiol is an acid whose pK is 5.1; thus at pH 8.0 it is over 99% ionized, and the absorption at 412 mμ can be used as a measure of mercaptan groups capable of displacing PNBP from the disulfide.

That 1 mole of *p*-nitrobenzenethiol is released per mole of mercaptan is shown by the following experiment. Various amounts of *p*-nitro-

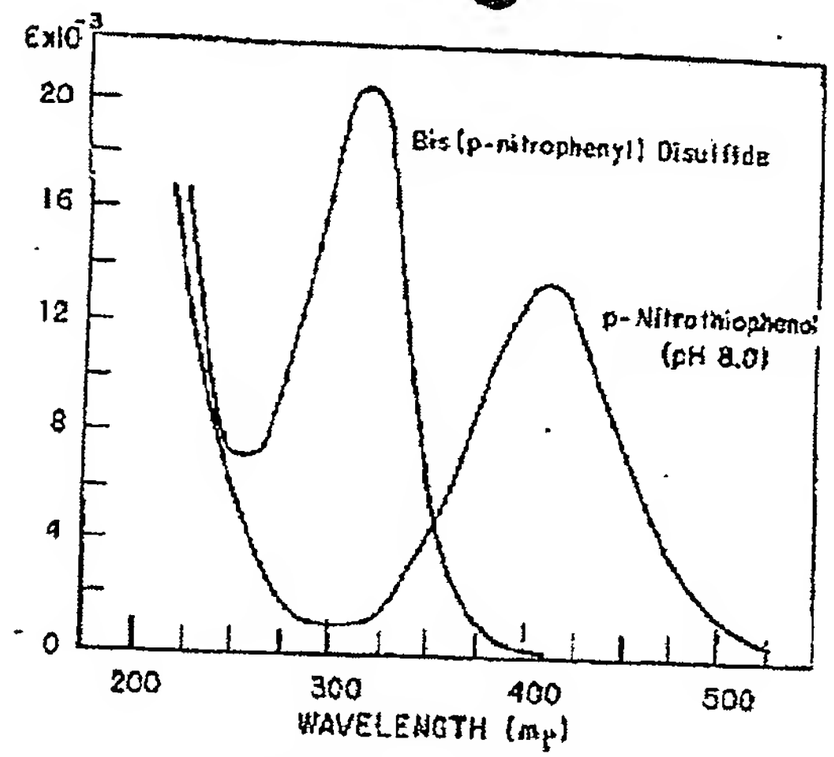


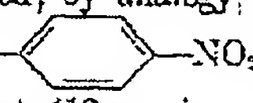
FIG. 1. Absorption spectra.

benzenethiol were measured into tubes, pH 8.0 phosphate buffer wa added, and the absorption was measured. Similarly, measured amount of 2-mercaptoethanol and glutathione were treated with bis(*p*-nitro phenyl)disulfide, and the absorbance was measured. The results ar shown below:

Compound	Concn. ($\times 10^{-5}$ *M*)	Aux	Extinction
p-Nitrobenzenethiol[a]	0.5	0.068	13,600
	1.0	0.135	13,500
	2.0	0.263	13,400
	3.0	0.411	13,700
	4.0	0.546	13,650
2-Mercaptoethanol[b]	1.0	0.135	13,500
	2.0	0.261	13,050
	3.0	0.412	13,700
	4.0	0.543	13,600
Glutathione[b]	1.0	0.138	13,800
	2.0	0.266	13,300
	3.0	0.408	13,600
	4.0	0.544	13,600

[a] Compound prepared as described under *Methods*.
[b] Solutions were standardized immediately before use by the method of Kimball *et al.* (3), and then diluted.

ince the extinction per mole is the same, whether the *p*-nitrobenethiol is obtained by dissolving the compound, or indirectly by proeing it from its dimer, it follows that 1 mole of *p*-nitrobenzenethiol released per mole of mercaptan.

The spectra of the disulfide (Fig. 1) show that it exhibits negligible sorption at 412 mμ. The addition of mercury or silver ions to a soluon of the *p*-nitrobenzenethiol at pH 8.0, or lowering the pH to 3.0, iminates the yellow color. Thus, whenever the aromatic anion is stroyed, the color is destroyed; by analogy, one would argue that a mixed disulfide (R—S—S—  —NO_2) does not absorb at 12 mμ. Thus, the absorption at 412 mμ is a direct measure of the itrobenzenethiol anion concentration.

Methods

Materials

1. *Bis(p-nitrophenyl)Disulfide (PNPD).* This can be purchased.[1] It should, owever, be recrystallized from glacial acetic acid, ethyl acetate, or both; m.p. 3–184°. This material can be irritating to some individuals, so it is well to avoid in contact with it. Exposure to sensitive areas may produce symptoms similar ivy poisoning.

2. *p-Nitrobenzenethiol* (for preparation of standard curves). Suspend commerrial material[2] in 5% sodium carbonate; filter into a well-stirred solution of hydrohloric acid (1 *N*). Filter. Recrystallize from glacial acetic acid: pale yellow crystals; m.p. 77–79°; soluble in acetone, alcohol.

Reagents

1. *PNPD* (10^{-1} *M*). Dissolve 30.8 mg. of bis(*p*-nitrophenyl)disulfide in 100 ml. f acetone.

2. *Buffer*. 0.1 *M* phosphate, pH 8.0.

Procedure

Mix. 4.0 ml. of unknown solution (should be neutral, relatively salt ree); 1.0 ml. buffer; 4.0 ml. acetone; and 1.0 ml. PNPD.

Determine the absorbance (*A*) at 412 mμ. A Beckman DU photoneter was used. The concentration of mercaptan (*C*) in the final solution is:

$$C = \frac{A}{13{,}600} \text{ moles/l.}$$

[1] Eastman Organic Chemicals: T1855.
[2] Aldrich Chemical Company, Inc.

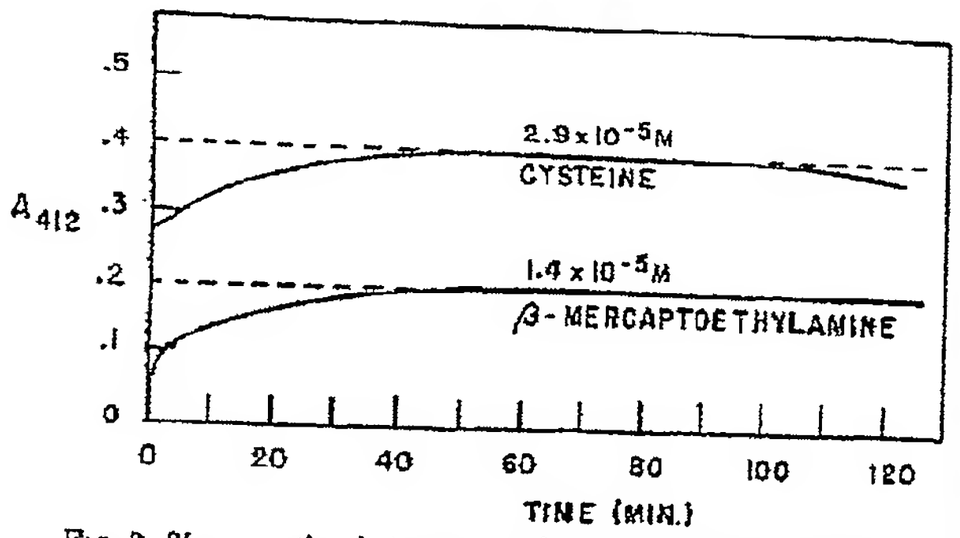


FIG. 2. Slow reaction between β-aminomercaptans and PNPD.

For simple mercaptans, the colored anion is produced immediately and is stable for at least 0.5 hr. For cysteine and related mercaptoethylamines, the full intensity is only developed after 60–90 min. (see Fig. 2).

RESULTS

Recovery

For the compounds that have been studied quantitatively (cysteine, 2-mercaptoethanol, and 2-aminoethanethiol), recovery is quantitative within the error of the absorption measurements (see Table I). It is clear that at low concentrations the variation in recovery is greatest.

TABLE I

Recovery of Mercaptans

Mercaptan	Amount added (μg./4 ml.)	Recovered	Per cent
2-Mercaptoethanol	20.1	20.3	101
	20.1	20.1	100
	20.1	20.2	101
	10.05	10.2	101
	10.05	10.0	99.5
	10.05	9.9	98.5
Cysteine	5.03	5.1	101
	5.03	5.0	99
	5.03	4.9	97.5
	1.10	.9	82
	1.10	1.0	91
	1.10	1.2	109

TABLE II

Compounds Treated with Bis(p-nitrophenyl) Disulfide

Color	No color
Cysteine	Methionine (10^{-1} *M*)
Dithiobiuret	Ethionine (10^{-1} *M*)
1-Dodecylmercaptan	Tetrahydro-1-methylthiophenium iodide (10^{-2} *M*)
Glutathione	2,2'-Hexamethylenebis(2-thiopseudourea)dihydrochloride (10^{-2} *M*)
2-Aminoethanethiol	6,6'-Thiodi-2-naphthol (10^{-4} *M*)
2-Mercaptoethanol	
Sodium sulfide	
Sodium sulfite	
Sodium thiocyanate	
Sodium thiosulfate	
Thioacetamide	
Thiopental	
Thiourea	

At these concentrations one is working at the limit of the sensitivity of the photometer. Thus, the lower practical limit is of the order of $2\text{–}4 \times 10^{-6}$ *M*, or about 1 μg. cysteine in 4 ml.

Specificity

The reaction used appears to be very specific for compounds containing a sulfur atom capable of existing as an anion at pH 8.0 (see Table II). No color was observed with pure samples of acids, amides, amines, alcohols, ketones, aldehydes, disulfides, sulfides, diazonium salts, amino acids, or esters at concentrations of 0.1–0.5 *M*; and none of these materials interfered with the reaction.

Interferences

It should be noted, however, that heavy-metal ions (mercury, silver, lead) do interfere under these conditions. The amount that will prevent liberation of the nitrothiophenol is approximately equal to the amount of mercaptan present; this indicates that the mercaptan anion does not exist as such in the presence of heavy-metal ions and that the Hg-S bond that is formed is not capable of reacting with the disulfide. This must be taken as further evidence for the specificity of the reaction; i.e., it is only between an RS^- species and the disulfide, at this pH.

Choice of pH

The choice of pH was governed by the facts that PNPD is slowly hydrolyzed at higher pH's, even though the percentage of mercaptan anion would be greater at higher pH's. At pH 8, the rate of appearance of color from PNPD alone is slow, but appreciable, being noticeable in 30–40 min. However, when it is necessary to measure the absorbance after longer intervals of time, this small hydrolysis color can be subtracted from that in the "unknown" solutions, and quantitative results are obtained.

Kinetics and Equilibria

Reaction (1) as written is very rapid with simple mercaptans (ethyl-, β-hydroxyethyl-, β-alkoxyethyl-, glutathione, sodium sulfide). The color formation at room temperature is complete in less than 10 sec., and the color remains constant for at least half an hour after formation. At that time, there is a slow disappearance of color, undoubtedly due to air oxidation of the nitrothiophenol.

While it has always been assumed that such a reaction as Reaction (1) is reversible, it is clear that for this reaction, at least, the position of equilibrium is far toward completion. For the mercaptans examined thus far, the yield of nitrothiophenol is 1 mole/mole mercaptan within the error of the absorption measurements. Attempts to study the reverse reaction are being made.

Application

This reaction has been used to study serum and blood mercaptan levels. For serum determinations, the following variation in method is applied.

	Blank	Test
Buffer	1.0 ml.	1.0 ml.
Water	5.9	5.9
Acetone	3.0	2.7
Reagent	0.0	0.3
Mix well and add		
Serum	0.1	0.1
Measure the absorbance at once.		

Mercaptan content of whole blood can be determined by the following variations:

Place into a large test tube (150 mm. × 25 mm.)

	Blank	Test
Water	5.0 ml.	5.0 ml.
Blood	0.010	0.010
Mix well		
Add: buffer	2.0	2.0
Acetone	3.0	2.7
Let stand at room temperature for 1 hr.		
Add: reagent	0.0	0.3
Measure the absorbance.		

Most bloods examined so far reached the equilibrium value in ½–1 min. Occasional samples of blood require 30–40 min. to come to equilibrium, so that several measurements of the absorbance may be desirable. Results of a series of such blood levels (see Table III) can best be ex-

TABLE III

Blood Mercaptan Concentrations

Blood	Concn. mM	Stand. dev.
GLE	19.4	1.6
	18.0	0.7
LCH	16.2	1.8
	15.6	0.1
PS	14.6	0.3
	15.1	0.4
	13.8	0.5
JAF*	10.0	0.1
	10.9	0.1
	11.8	0.7
DLD	15.8	1.2
	13.4	1.5
WBN	15.0	0.5
DL*	11.6	1.0
	11.7	1.4
RCR	17.7	1.0
	14.2	4.2

* Female.

pressed as the millimolar concentration of mercaptan, since the average molecular weight of the blood mercaptans is not known.

$$C = \left(\frac{A_{412}}{1.36 \times 10^{-4}}\right)(10^3)(10^3 mM)$$
$$= 736 A_{412}(mM)$$

Summary

1. A method for the determination of mercaptans is described. It depends on the interchange of bis(*p*-nitrophenyl)disulfide and mercaptan anions at pH 8.0.

2. A study of the specificity of the reaction indicates that it occurs only with the S^- group (actual or potential, as in thiourea).

3. Heavy-metal ions interfere with the reaction by irreversibly bonding any S^- ions.

4. The kinetics of the reaction with cysteine and β-mercaptoethylamine were studied.

5. Several applications of this reaction were made: (*a*) the determination of mercaptan in synthetic compounds; (*b*) mercaptan reactivity in proteins; and (*c*) determination of blood and serum concentrations.

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 74, 451–457 (1958)

The Action of Some Diamine Optical Antipodes on Acetylcholinesterase Inhibition and on Conduction in Desheathed Bullfrog Sciatic Nerve[1]

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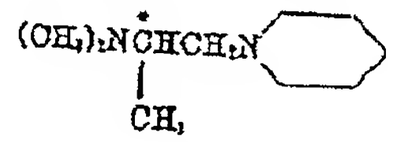
Introduction

Previous study (1) of the acetylcholinesterase (AChE) inhibitory properties of the racemic diamine I revealed a moderate strength in



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competitive inhibition of the enzyme–acetylcholine system, roughly one order of magnitude greater than that shown by choline. Parallel study (2) of the ability of DL-I to block conduction in frog sciatic nerve, and comparison with eserine as a standard blocking agent, pointed to an order of strength greatly in excess of that predicted from its *in vitro* power against isolated electric eel AChE, with the further observation that its blockade of the propagated action potential at 10 m*M* concentration appeared to be irreversible to some extent. Recovery of the drug-exposed nerve on washing was found to become progressively poorer as the contact time with the drug solution was increased.

Further probing of the fine structure of the catalytic surface of AChE has been attempted by a resolution of the diamine I into its (+)- and (−)-optical antipodes, with each being tested separately and in com-

[1] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

ATTACHMENT C

Direct Colorimetric Assay of Free Thiol Groups and Disulfide Bonds in Suspension of Solubilized and Particulate Cereal Proteins[1]

KIN-YU CHAN and BRUCE P. WASSERMAN[2]

ABSTRACT

A direct colorimetric method that simultaneously combines measurement of solubilized and insoluble thiol groups and disulfide bonds in corn meal-based materials is described. Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid), which reacts specifically with thiol groups, or disodium 2-nitro-5-thiosulfobenzoate, which reacts with cysteine and thiol groups formed after reduction of disulfide bonds with sodium sulfite, were reacted directly with corn meal in the presence of surfactants (urea and/or sodium dodecyl sulfate), releasing the soluble chromophore 2-nitro-5-thiobenzoate. After a clarification step to remove suspended material, absorbance at 412 nm was read. This assay was highly reducible, and measurements agreed with direct amino acid analysis. T screw extrusion of corn meal at 150° C at moisture levels of 16 and had no significant effect on cysteine or disulfide bond levels. Other pos changes such as disulfide bond rearrangements could not be determ by the mixed-phase assay. This method provides a rapid and conver means for screening thiol and disulfide levels in insoluble proteinac materials.

Cereal Chem. 70(1):2

Disulfide bonds are thought to play an important role in the texture of cereal-based products. However, because of the hydrophobic and insoluble nature of cereal proteins, quantification of thiol and disulfide bonds has proven difficult. Complete extraction of corn meal protein with sodium dodecyl sulfate (SDS), urea, and a reducing agent leads to cleavage of disulfide bonds. Furthermore, since extraction with SDS and urea results in only partial solubilization of the protein, assays based on solubilization followed by measurement of thiol and disulfide content often yield highly variable results and underestimates of true thiol and disulfide group content. A technique that avoids this initial protein solubilization step would largely eliminate these problems.

This article describes a solid-phase assay that simultaneously combines quantification of soluble and particulate thiol and disulfide groups in cereal-based proteins. The principle of this method is to suspend the entire sample in urea and to react it with a color reagent that will simultaneously react with both soluble and insoluble proteins, with release of a soluble chromophore. Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), which reacts specifically with thiol groups (Ellman 1958, Riddles et al 1983), and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$), which is used to quantify disulfide group content (Thannhauser et al 1987), are ideally suited for this purpose, since reaction with either results in the release of the 2-nitro-5-thiobenzoate anion (NTB^{2-}), which is soluble in aqueous solution. Following the removal of insoluble material by clarification steps, absorbance at 412 nm is then read. This method was used to assess the effects of twin-screw extrusion processing on thiol and disulfide levels in corn meal.

MATERIALS AND METHODS

Materials

Corn meal (12% moisture, 7% protein, 0.7% oil, 0.5% fiber, 0.4% ash, and 79.4% N-free extract) was obtained from Lauhoff Grain Co., Danville, IL. DTNB and $NTSB^{2-}$ were obtained from Aldrich Chemical Co., Milwaukee, WI. Extrusion was conducted in a Brabender type 2003 single-screw extruder with an axially grooved barrel; length-to-diameter ratio, 20; screw diameter, 1.9 cm (0.75 in.); screw length, 38.1 cm (15 in.); and compression ratio, 1:3.

Solid-Phase Assay for Free Thiol Content

Colorimetric reactions were conducted under the conditions described by Ellman (1958, 1959). Unless otherwise indicated, samples (30 mg ground to 40 mesh and dried in vacuo) were suspended in 1.0 ml of reaction buffer consisting of 8*M* urea, 10 m*M* DTNB, 3 m*M* ethylene-diaminetetraacetic acid (EDTA), and 0.2*M* Tris-HCl, pH 8.0. SDS (1%) was present where indicated. Samples were incubated under N_2 for various intervals. To remove particulate matter, samples were centrifuged at 13,600 × *g* for 10 min in a microcentrifuge. A 0.1-ml aliquot of supernatant was removed and diluted with 0.9 ml of 8*M* urea, 1% SDS, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. This solution was centrifuged at 13,600 × *g*, and its absorbance was read at 412 nm.

Solid-Phase Assay for Total Sulfhydryl Group Content

Colorimetric reactions were conducted under the conditions described by Thannhauser et al (1987). Unless otherwise indicated, samples (30 mg ground to 40 mesh and dried in vacuo) were suspended in 1.0 ml of reaction buffer consisting of 8*M* urea, 0.1*M* sodium sulfite, 3 m*M* EDTA, 0.2*M* Tris-HCl, pH 9.5, and 10 m*M* $NTSB^{2-}$, synthesized from DTNB in the presence of sodium sulfite and O_2 as described in Thannhauser et al (1987). SDS (1%) was added where indicated. To remove particulate matter, samples were centrifuged at 13,600 × *g* in a microcentrifuge for 10 min. A 0.1-ml aliquot of supernatant was removed and diluted with 0.9 ml of 8*M* urea, 1% SDS, 0.1*M* sodium sulfite, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. This solution was centrifuged at 13,600 × *g*, and its absorbance was read at 412 nm. Disulfide group content was calculated as the difference between thiol group content before and after reduction of disulfide bonds with sulfite. Total cysteine was calculated as [-SH] + 2[-S-S-].

Two-Step Method for Sulfhydryl-Disulfide Assay

Unless otherwise indicated, samples (60 mg ground to 40 mesh and dried in vacuo) were extracted under N_2 with 2.0 ml of buffer consisting of 8*M* urea, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. On the basis of a time course of protein solubilization (Fig. 1A), extractions were conducted for 3 hr under N_2. To remove particulate matter, samples were centrifuged at 16,000 × *g* for 30 min in a Sorvall RC5C preparative centrifuge (Sorvall Instruments, Du Pont, Wilmington, DE). Aliquots of 0.2 ml of supernatant were removed and brought to 1.0 ml with appropriate DTNB- and $NTSB^{2-}$-containing reaction mixtures (see above) for spectrophotometric determination of thiol and disulfide groups.

RESULTS

Development of the Method

Protein solubility. With insoluble proteins, the extent of protein solubilization is dependent on solvent composition, exposure to O_2, and solubilization time. Figure 1 shows time courses of corn

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meal protein solubilization that confirm that maximal solubilization is obtained only when reducing agents are added. Furthermore, solubilization is enhanced when O_2 is excluded (Fig. 1). On the basis of Kjeldahl assays of protein remaining in the pellets, even under optimal conditions (urea, SDS, and β-mercaptoethanol combined) we found that only 85% of the protein could be solubilized. The pH of the solubilization mixture was varied between 7 to 10, and similar levels of protein were solubilized over this range. Therefore, to directly assay for thiol and disulfide levels present in the entire sample and to avoid tedious fractionation procedures, the direct colorimetric assay was developed.

Chemical and physical aspects of the direct colorimetric assay. A schematic of the mixed-phase method is shown in Figure 2. The key feature is that regardless of whether DTNB reacts with soluble or insoluble protein, the product of this reaction, the chromophore NTB^{2-}, is soluble in aqueous solution. NTB^{2-} gives a yellow color with an extinction coefficient of $13{,}600 M^{-1}$ cm^{-1} at 412 nm. These reactions are conducted in the presence of urea or urea and SDS to maximize the reactivity of any thiol or disulfide groups that may be buried within the hydrophobic protein matrix. In samples analyzed in tandem, disulfide bonds are determined indirectly with $NTSB^{2-}$ (Thannhauser et al 1987), which is added to protein suspensions with sodium sulfite. At pH 9.5, sodium sulfite completely cleaves disulfide bonds. One of the sulfur atoms forms a sulfated derivative, whereas the other forms the thiolate anion, which, with thiol groups from cysteine, reacts with $NTSB^{2-}$. Thus, $NTSB^{2-}$ quantifies total thiol and disulfide content, and disulfide content is then calculated by difference. Corn meal did not contain high enough levels of compounds that absorbed at 412 nm to interfere with measurement of NTB^{2-}.

Clarification of protein suspensions. To complete this procedure, a clarification step must be conducted to remove suspended particles that would otherwise interfere with obtaining an accurate colorimetric reading. Various combinations of four procedures were evaluated (Table I): 1) centrifugation at 13,000 $\times$ *g* in a preparative centrifuge; 2) centrifugation at 13,000 $\times$ *g* in a microcentrifuge; 3) spin filtration through a 1-ml bed of glass wool in a microcentrifuge at 13,000 $\times$ *g*; and 4) dilution of the reaction mixture by 1:10, followed by centrifugation at 13,000 $\times$ *g* in a microcentrifuge.

The combination of all four steps resulted in complete clarification, but the process was time-consuming and labor-intensive. The combination of steps 2 and 4, microcentrifugation and dilution followed by a second microcentrifugation, proved most rapid and gave complete clarification (trial 5, Table I). This condensed procedure produced results equivalent to that of the four steps combined and was therefore incorporated as part of the standard procedure.

Time course and concentration dependence. Figure 3 shows time courses of both colorimetric reactions with corn meal. By 20 min or even less, each of the reactions reached completion. These time courses also demonstrated that NTB^{2-} is stable for at least 1 hr.

Figure 4 shows that absorbance was linear with the amount of sample. It should be noted that above 40 mg, assay mixtures would become viscous and difficult to dilute during the clarification step. Below 10 mg, increased weighing errors were apparent. On the basis of these considerations, a suitable sample weight range for corn meal is between 10 and 40 mg.

Comparison to literature values. As a positive control, the thiol and disulfide group content of lysozyme was determined. In SDS and urea the thiol content of lysozyme was measured to be 0.35 mol/mol and the disulfide content 3.87 mol/mol. These compared favorably with the literature values of 0 mol/mol and 4 mol/mol, respectively (Phillips 1974). SDS was critical. Without it,

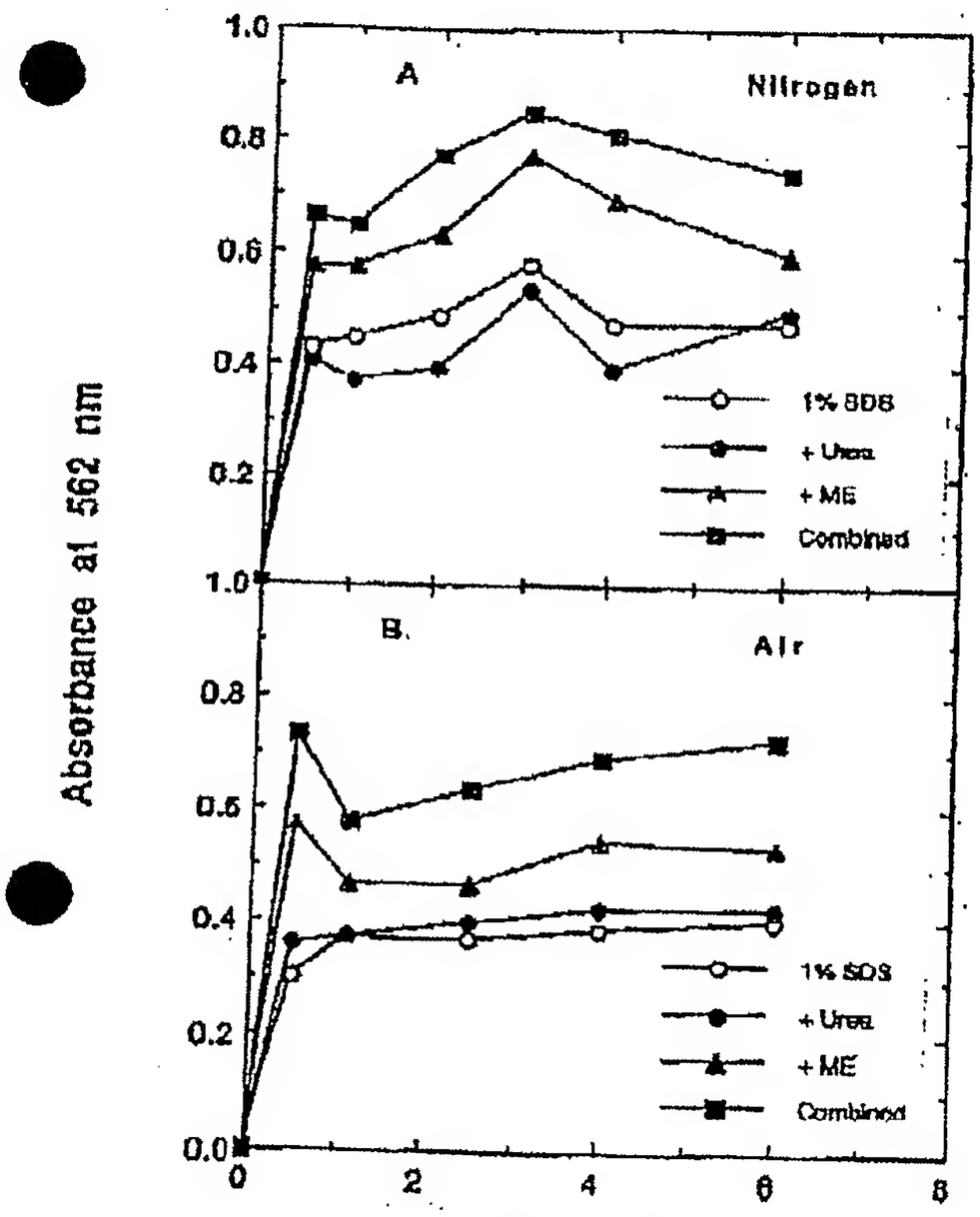

Fig. 1. Time course for protein solubilization from corn meal under nitrogen (A) and air (B). Native corn meal (60 mg) was solubilized at room temperature in 2 ml of 1% sodium dodecyl sulfate (SDS), 1% SDS and 8 *M* urea, 1% SDS and 2% β-mercaptoethanol (ME), or all combined in 12.5 m*M* sodium tetraborate buffer (pH 10.0) for various times as indicated. The samples were then centrifuged at 14,500 $\times$ *g* for 30 min, and aliquots ranging from 10 to 30 μl were transferred to a microcentrifuge tube and mixed with 1 ml of water. Soluble protein was assayed colorimetrically by the bicinchoninic acid assay with trichloroacetic acid precipitation (Brown et al 1989).

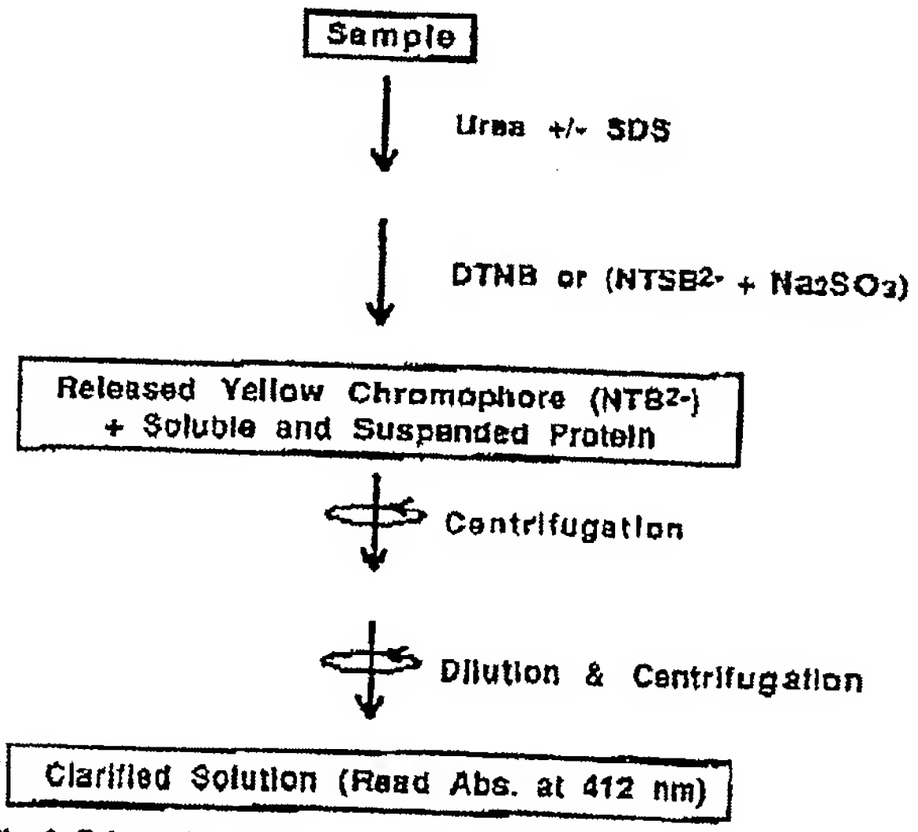

Fig. 2. Schematic of the direct colorimetric assay for thiol and disulfide groups. Samples were suspended in urea and/or sodium dodecyl sulfate (SDS) and reacted directly with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) or disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$). Clarification was conducted to remove suspended particles that would otherwise interfere with an accurate colorimetric reading.

TABLE I
Effects of Clarification Steps on Absorbances of 5,5′-Dithiobis (2-Nitrobenzoic Acid) (DTNB) and Disodium 2-Nitro-5-Thiosulfobenzoate ($NTSB^{2-}$)

Trial	Clarification Steps[a]				Absorbance, 412 nm	
	Preparative Centrifugation[b]	Microcentrifugation[c]	Spin Filtration[d]	Dilution and Microcentrifugation[e]	DTNB	NTS
1	−	−	−	−	1.212	1.65
2	+	+	+	+	0.093*[f]	0.34
3	+	+	−	+	0.099*	0.36
4	−	+	+	+	0.084*	0.34
5	−	+	−	+	0.084*	0.35
6	+	−	+	+	0.102*	0.35
7	+	−	−	+	0.122	0.35
8	−	−	+	+	0.093*	0.31
9	+	+	+	−	0.284	0.41
10	+	+	−	−	0.248	0.45
11	−	+	+	−	0.170	0.44
12	−	+	−	−	0.160	0.49
13	+	−	+	−	0.262	0.49
14	+	−	−	−	0.480	0.66
15	−	−	+	−	0.203	0.40
16	−	−	−	+	0.143	0.36

[a] +, Step used; −, step not used.
[b] Centrifugation at 16,000 × *g* for 30 min in a Sorvall centrifuge (model RC5C, Sorvall Instruments, Du Pont, Wilmington, DE).
[c] Microcentrifugation at 13,600 × *g* for 10 min using a Fisher microcentrifuge (model 235A, Fisher Scientific, Pittsburgh, PA).
[d] Conducted by adding 0.5 ml of sample to a 0.5-ml microcentrifuge tube filled with glass wool with a hole punched in its bottom. This was inser into a 1.5-ml microcentrifuge tube and centrifuged for 3 min at 13,600 × *g*. The clarified solution was recovered from the lower tube.
[e] Tenfold dilution of the clarified solution from each step described above with either 8*M* urea, 1% sodium dodecyl sulfate (SDS), 3 m*M* ethyle diaminetetraacetic acid (EDTA), and 0.2*M* Tris-HCl, pH 8.0, for thiol group assay, or with 8*M* urea, 1% SDS, 0.1*M* Na_2SO_3, 3 m*M* EDT and 0.2*M* Tris-HCl, pH 9.5, for disulfide group assays. Dilution was followed by microcentrifugation (13,600 × *g*).
[f] Asterisks signify the absence of turbidity, defined by the lowest absorbance values.

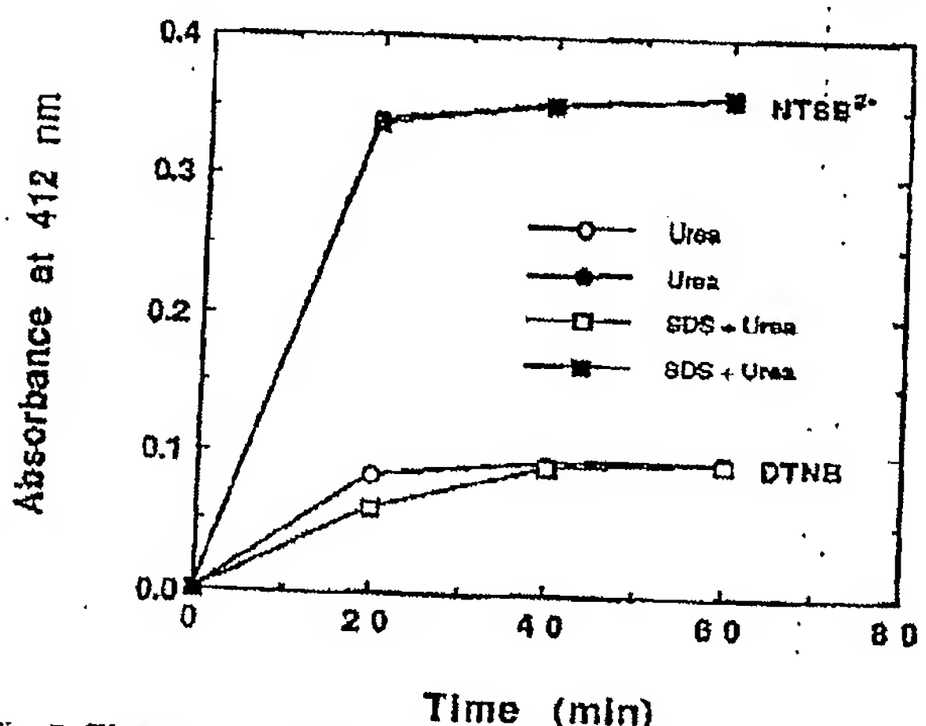

Fig. 3. Time courses of colorimetric reactions with corn meal. Samples were incubated at room temperature in 8*M* urea and 1% sodium dodecyl sulfate (SDS) and 8*M* urea with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) as described in Materials and Methods.

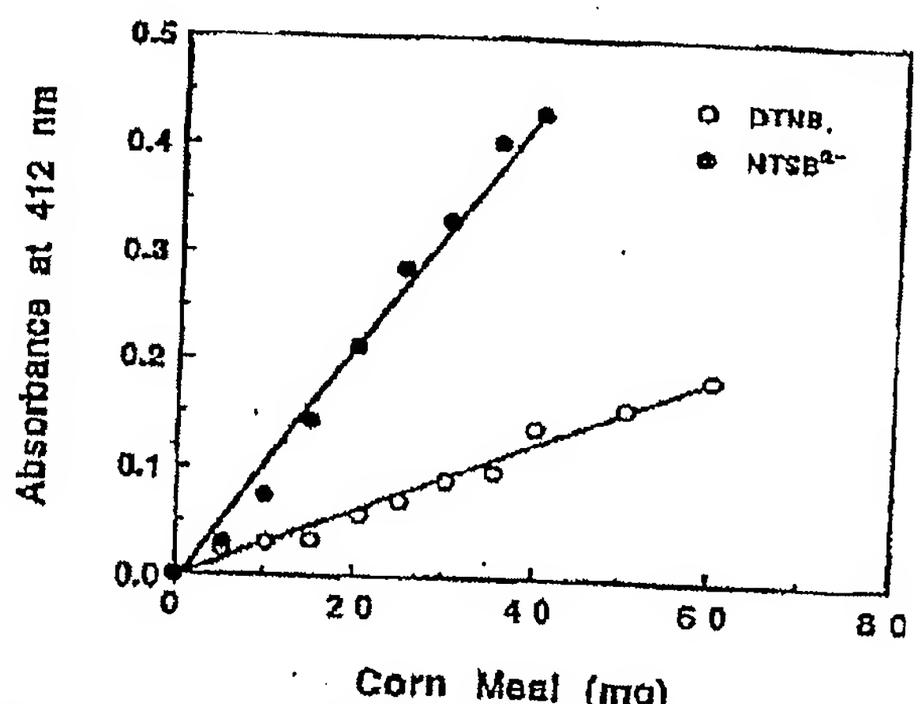

Fig. 4. Concentration dependence of the colorimetric reactions. Corn meal was incubated at room temperature in 8*M* urea with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) and assayed as described in Materials and Methods.

the disulfide content was only 0.65 mol/mol, implying that thiol groups in lysozyme are buried and must be exposed before addition of color reagents. The total cysteine content of corn meal was 196.4 nmol/mg of protein, comparing favorably with the literature value of 199.7 nmol/mg (Lasztity 1984).

Comparison of the direct colorimetric assay with the two-step method. The direct colorimetric assay was compared with a two-step assay. In the latter, protein was first solubilized from samples with urea and SDS. After centrifugation, an aliquot of the supernatant was analyzed for free and total cysteine content with DTNB and $NTSB^{2-}$. Two major differences between each assay were observed (Table II). First, higher readings were obtained by the direct colorimetric assay. Most likely, this is due to the fact that some of the protein resists solubilization in SDS and urea, and it is therefore less available to react with DTNB and $NTSB^{2-}$ in the two-step assay. Second, the standard error was alway significantly higher in the two-step assay. This increased variabilit also can be attributed to the incomplete release of protein durin the solubilization step.

Application of the Method

Effect of extrusion on the thiol and disulfide group conten of corn meal. The effect of twin-screw extrusion on the thiol and disulfide content of corn meal was determined. Table III shows that in the presence of 8*M* urea, extrusion caused an apparent 25–30% increase in free thiol content relative to that of the control. This was accompanied by a small decrease in disulfide groups. At most, only about 5% of the total cysteine content was affected, suggesting a small amount of extrusion-induced

rupturing of disulfide bonds. Total cysteine content did not change as the result of extrusion, indicating that significant oxidation or heat damage did not occur.

An observation of possible significance was decreased reactivity of thiol and disulfide groups after extrusion when SDS was added to reaction mixtures in the presence of 8*M* urea (Table III and Fig. 5). Figure 5A illustrates that addition of increasing levels of SDS to native corn meal had no effect on reactivity with either DTNB or $NTSB^{2-}$. However, in extrudates, addition of SDS at levels as low as 0.1% caused a significant (30%) decline in reactivity with both color reagents. (Fig. 5B and C). In light of the presumption that SDS, a powerful denaturant, would be expected to enhance the reactivity of thiol and disulfide groups found at the surface or buried within hydrophobic proteins, this finding was unexpected. Because SDS did not decrease the reactivity of native corn meal, it is unlikely that this decline is due to interference with the color reactions.

DISCUSSION

The direct colorimetric assay is a rapid and convenient method for quantifying the cysteine and cystine content of suspensions that contain a mixture of soluble and insoluble proteins. Here, assays were conducted directly on corn meal using the color reagents developed by Ellman (1958, 1959) and Thannhauser et al (1987). To maximize reaction of the protein with DTNB and $NTSB^{2-}$, surfactants (combinations of urea and SDS) were added to reaction mixtures. A clarification step was added to remove suspended material before reading of absorbance at 412 nm. The critical feature of this assay is that the NTB^{2-} chromophore is released into solution and does not covalently bind to protein.

With the exception of some radioisotopic (Schofield et al 1983) and amperometric (Redman and Ewart 1971) methods and direct amino acid analysis (Ewart 1988), most previous attempts to quantify thiol and disulfide content of insoluble samples required extraction of protein with denaturants and reducing agents and removal of the reducing agent before assay by colorimetric (Burgess and Stanley 1976, Hager 1984, Abe et al 1985) or other (Draper and Catsimpoolas 1978, Neumann et al 1984, Alary and Kobrehel 1987) means. The semidirect method described by Schofield et al (1983) for detection of thiol groups in gluten was based on the reaction of gluten with ^{14}C-iodoacetamide. Nevertheless, quantification of incorporated radiolabel required subsequent fractionation steps. The amperometric assay of Redman and Ewart (1970) was conducted directly on glutelin suspended in guanidine HCl. Incomplete extraction of protein and losses during fractionation steps could have led to significantly underestimated values for thiol and disulfide group content. Two notable differences observed between the direct solid-phase and two-step

TABLE II
Comparison of the Direct Colorimetric Assay with a Two-Step Method[a]

Method	Sulfhydryls	Disulfides	Total Cysteine
Direct colorimetric	2.29 ± 0.08	6.28 ± 0.09	14.86 ± 0.20
Two-step[b]	2.09 ± 0.45	3.29 ± 0.73	8.68 ± 1.52

[a] Mean ± standard error of values for five replicates. Values are nanomoles per milligram of sample.
[b] The two-step assay, analogous to Hager (1984), consisted of solubilization in 1% sodium dodecyl sulfate and 8*M* urea, followed by centrifugation and assay of an aliquot of supernatant for thiol and disulfide group content (see Materials and Methods).

TABLE III
Effect of Twin-Screw Extrusion on the Free Thiol and Disulfide Group Content of Corn Meal[a]

Extrusion Conditions					
	Temperature (°C)	H_2O (%)	Thiol	Disulfide	Total Cysteine
In 8*M* urea					
Native			31.0 ± 0.9	82.7 ± 1.1	196.4 ± 2.4
2/13-2	151	16.3	40.4 ± 1.2	80.3 ± 2.2	200.9 ± 4.6
3/13-1	152	18.2	38.2 ± 2.5	81.2 ± 3.7	200.6 ± 7.9
In 8*M* urea + 1% sodium dodecyl sulfate					
Native			30.4 ± 1.1	83.2 ± 1.3	196.9 ± 2.8
2/13-2	151	16.3	22.9 ± 2.2	77.6 ± 3.0	178.0 ± 6.3
3/13-1	152	18.2	25.4 ± 1.5	77.0 ± 2.7	179.4 ± 5.5

[a] Mean ± standard error of values for three replicates. Values unless otherwise noted are nanomoles per milligram of protein.

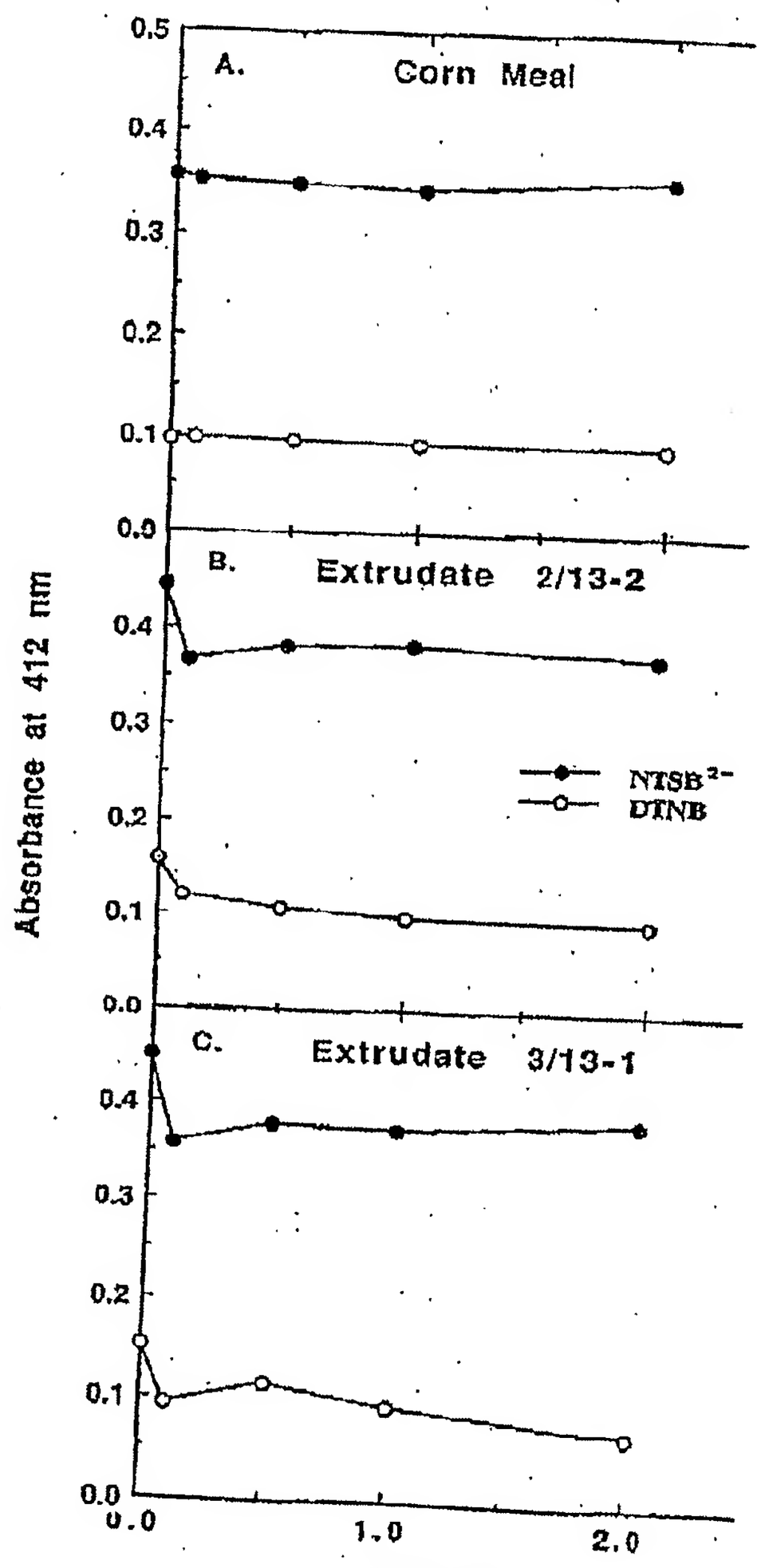

Fig. 5. Effect of sodium dodecyl sulfate (SDS) on reactivity with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$). Native corn meal and extrudates 2/13-2 and 3/13-1 (A–C, respectively) were incubated at room temperature in 8*M* urea with 0–2% SDS with DTNB and $NTSB^{2-}$ and assayed as described in Materials and Methods.

methods (Table II) are that higher readings were obtained by the direct colorimetric assay because there is no opportunity for protein to be lost, and that standard errors were always significantly higher in the two-step assay.

The solubility of cereal proteins is strongly affected by reducing agents and O_2. The effect of O_2 in the solubilization time course was an observation of note (Fig. 1). In both the absence (Fig. 1A) and presence (Fig. 1B) of O_2, the first 30 min was characterized by a rapid release of protein. Under N_2, solubility increased in the presence of reducing agent for up to 3 hr (Fig. 1A); in air, protein solubility markedly declined between 30 min and 1 hr (Fig. 1B). Furthermore, total extractability is improved by exclusion of O_2, with about 12% more protein solubilized (Fig. 1A at 3 hr vs. Fig. 1B at 30 min). Since the rapid decline in solubility after 30 min in air (Fig. 1B) did not occur in the absence of reducing agent, the involvement of cysteine is likely. O_2 appears to promote reoxidation of thiols to disulfide bonds, thus causing some of the solubilized protein to precipitate.

The direct colorimetric technique was used to determine the effect of twin-screw extrusion on the thiol and disulfide content of corn meal. Table III suggests that a small amount of extrusion-induced rupturing of disulfide bonds occurred; however, this was statistically insignificant at the $P = 0.1$ level, with 5%, at most, of the cysteine content affected. These results are in accordance with Schofield et al (1983), who found that the free thiol group content was unaffected by heating at 100°C. In contrast, our results differ from the findings of Burgess and Stanley (1976) and Hager (1984), who found slight decreases in disulfide content but major increases in thiol groups in soy concentrate (70% protein on a dry basis) extruded at >180 and <150°C, respectively. Since the direct colorimetric technique does not measure disulfide bond rearrangements, the extent to which these may occur cannot be ascertained.

The unexpected but significant decrease in reactivity of both thiol and disulfide groups of extruded corn meal upon addition of SDS to the reaction mixtures (Fig. 5) suggests that extrusion-induced conformational changes in protein structure may inhibit access of colorimetric reagents when SDS is present. One possible explanation for this finding is that conformational changes in protein structure inhibit access of these reagents when SDS is present. Alternatively, the cysteine in extrudates is more prone to oxidation after extrusion. The decrease in total cysteine (197 vs. 179 nmol/mg of protein in native and extruded corn meal, respectively; Table III) is consistent with this possibility.

Although this study focused specifically on corn meal, the direct colorimetric assay should be applicable to a wide range of protein-containing products.

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